Final report-

Award Number 2006-DN-BX-K018

Repair of Damaged DNA for Forensic Analysis

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10/28/2008

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Abstract:

Current DNA-based forensic analysis methods rely on Polymerase Chain Reaction (PCR) to amplify predetermined regions of genomic or mitochondrial DNA for comparative analysis, scoring variations in the size of the amplified DNA segments. While this works extremely well for fresh or well-preserved DNA samples, it is common for the DNA in unprotected samples to fail amplification because it has been damaged. As a highly complex molecule, DNA can be damaged in a number of ways, resulting in breaks in the strands or removal or chemical alteration of the nucleotide bases. The severity of damage to DNA can vary, but once there are one or more lesions in the DNA strands within the segment to be amplified by PCR, the amplification and analysis will fail. Fortunately, DNA is double-stranded and so redundantly structured, and nature has devised a rich collection of mechanisms to repair many kinds of damage, often using the information in one strand to reconstruct the other. In principle, repairing the lesions in the DNA strands will allow those samples to function in a standard CODIS panel STR analysis.

In this program, GE Global Research has developed a DNA repair method that can be used on damaged DNA samples. The repair method utilizes a mixture of DNA repair enzymes to repair or replace damaged strand segments in subject DNA. The method is designed to function after DNA isolation and quantification, but prior to DNA amplification and STR analysis. If utilized, this DNA repair method should allow forensic investigators to examine samples to their fullest potential, ultimately leading to more samples having accurate, usable results. With ultimate commercialization, the proposed method will allow forensic investigators to overcome the limitations of current DNA technology, more fully utilize the evidence that is available in criminal cases regardless of mild to moderate environmental damage, and ultimately lead to more successful investigation and prosecution of criminal matters.

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Executive Summary:

The Problem:

Current DNA-based forensic analysis methods rely on Polymerase Chain Reaction (PCR) to amplify predetermined regions of genomic or mitochondrial DNA for comparative analysis, scoring variations in the size of the amplified DNA segments. While this works extremely well for fresh or well-preserved DNA samples, it is common for the DNA in unprotected samples to fail amplification because it has been damaged. Fortunately, DNA is double-stranded and so redundantly structured. This report will describe the work done towards developing a repair method that utilizes a mixture of DNA repair enzymes to either repair or replace damaged strand segments in subject DNA.

We have developed a DNA repair method which can be used by forensic investigators when DNA samples are suspected of being environmentally damaged. This will allow for more complete characterization of forensic evidence to be used in criminal investigation DNA analysis performed at forensic laboratories today is confounded by DNA samples that have been exposed to uncontrolled environmental conditions. This damage frequently results in an insufficient number of loci, therefore, not allowing for match comparisons. Repairing the DNA strands has been shown in this work to allow a greater number of alleles to be amplified, increasing the statistical significance and allowing for match comparisons to controls.

Background:

Typically, environmentally damaged samples contain either degraded DNA fragments (double stranded breaks in the DNA) or contain somewhat intact DNA with single stranded nicks and DNA lesions[i]. Some examples of damaging exposure include:

- Acid and heat exposure, which leads to missing bases, or apurinic and apyrimidinic sites (AP sites)
- Ionizing radiation and electrophilic agents, including alkylating agents, which modify bases or the sugar backbone
- Oxidative damage (very common), which leads to altered bases
- UV irradiation, which produces cyclobutane dimers

Both degraded (small molecular weight) and base-modified DNA samples fail with standard analysis methods, however, for different reasons. PCR-based analysis is essentially a proximity assay which functions to amplify DNA that contains both of two predefined sequences (primer binding sites). When the samples have been degraded to a size smaller than the distance between the two sequences being interrogated, the analysis will fail. Conversely, when the sample is intact, but contains numerous base modifications (lesions) to the DNA that cannot be "read" by the amplification enzyme, the sample will also fail to analyze correctly. The key difference between these two observations is that base- modified samples have the potential to be repaired. One strategy being pursued recently to overcome damage involves developing methods that can utilize badly damaged samples [ii, iii, iv], however, we feel that methods which *fix* damaged samples could only complement these strategies.

Research Design and Findings:

Damaged DNA contains nucleotide modifications in the DNA strands that are a block to the standard DNA replication machinery of the cell [v]. As a result, nature has developed cellular pathways designed to identify damaged DNA and eliminate it while preserving the competence of the genome. These naturally occurring pathways are roughly broken into four categories:

1) Ligation to seal nicks,
2) Direct reversal of the modification to normal state,
3) Recombination repair using a second DNA copy as template, and
4) Excision repair [vi].

With the exception of recombination repair, GE Global Research has investigated the possibility of using commercially-available enzymes, which function in these pathways, to repair damaged DNA prior to amplification and analysis.

The main goal of the proposed program was to develop a method to repair DNA and determine its optimal method of use in forensic analysis. This method uses an enzyme blend designed to fix environmentally-damaged samples that have DNA analysis issues. The method is designed to function after DNA isolation and quantification, but prior to DNA amplification. It is not anticipated that the method will be used on precious samples, but rather samples that are not limiting and have already yielded poor results using standard forensic methods. The underlying hypothesis is that there is an "opportunity window" in the degradation of DNA during which the sample has not been completely degraded, yet contains a high enough concentration of base modifications (which present a block to replication by DNA polymerase) to prevent the robust amplification of the loci needed for STR sizing by PCR. This being the case, it should be possible to prepare an enzymatic solution capable of repairing the remaining DNA.

For DNA repair, three classes of enzyme can be used. The first is direct repair of the damage. This includes DNA polymerase, which fills in gaps leaving single stranded DNA nicks, ligase, which seals nicks in the double stranded template, and photolyase, which catalyses the reversal of certain light induced damage. The second class are enzymes that identify base-damaged nucleotides and simply remove the base section of the nucleotide (glycosylase activity) leaving an intact sugar backbone. This product is referred to as an apurinic or apyrimidinic site (AP site) and is itself a block to replication. In vivo, AP sites are removed by AP endonucleases. In some cases, the glycosylase is associated with an AP endonuclease and both steps are accomplished by one enzyme. In other cases an AP endonuclease is needed in addition to a DNA glycosylase to complete the base removal process. The product of this removal can be either nicked DNA with the AP site still associated with the strand, or a single base gap with the AP site eliminated, and in either case are still blocks to replication. Finally, there is nick translation. In this process, a DNA polymerase with the ability to both replicate DNA and simultaneously remove blocking single stranded DNA (5'-3' DNA polymerase and 5'-3' exonuclease activities) is utilized to move down a strand of DNA, essentially eliminating damaged bases (e.g. AP site) in front of it while synthesizing an undamaged version. In this work

we utilize E coli DNA polymerase I, which also contains an intrinsic 3'-5' proofreading exonuclease activity that can remove damaged bases (e.g. AP sites) that are located at the 3' end of a nick on double stranded DNA, which is one possible product of a glycosylase. When finished, the enzyme dissociates, leaving a nick that can be sealed with ligase. This repaired DNA has a higher likelihood of amplification of STR loci, and therefore, obtaining a more complete STR genetic profile.

GE Global Research developed an optimized mix of the enzymes (DNA ligase, various glycosylases and AP endonucleases, and DNA polymerase) that can be added to DNA containing any of a variety of damaged bases. After a simple incubation (during which the damage to the DNA is identified, removed, and DNA is sealed) the resulting sample is moved to a variety of DNA analysis procedures including PCR analysis of STR loci.

During the project we first developed a model system for testing the repair reaction using specifically damaged human DNA samples. These DNA samples were damaged using heat and acid treatment, UV irradiation, alkylation, or oxidation. These samples were then tested in PCR reactions designed to amplify each of the CODIS loci separately. The PCR reaction on these loci are meant to mimic the STR testing that would occur in an actual forensic laboratory. While the PCR reactions on these 14 loci are performed individually instead of a multiplex reaction, the observed results when amplifying a nanogram of DNA that has been damaged is similar to that observed in a forensic laboratory. In general, as the amount of damage was increased, the number of loci that failed to amplify increased, as expected. The damaged samples that gave partial profiles, meaning that only a subpopulation of the 13 loci correctly amplified, consistently did so. DNA samples were made that were damaged to the extent that each only gave a partial profile. These control samples were then used to optimize the enzyme mixtures and method of using the mixture for repair. Each mixture was evaluated on its ability to improve the number of loci that correctly amplified (as compared to the untreated control sample).

We determined that with each type of damage induced, as the samples were damaged, the DNA was concurrently degraded. But as we had predicted, the native molecular weight of the damaged DNA was consistently higher than the denatured molecular weight. Meaning that as the DNA was being modified to contain lesions that might be repairable, it was also being nicked (as indicated by the decrease in denatured molecular weight). This indicated that while there were double stranded DNA breaks being formed, which can not be repaired by our methods, the resulting double stranded segments contained nicks. It is important to note this result, as it is almost certain that this same observation will be the case with actual DNA evidence that is damaged by exposure. It is also important to note that if these samples are *degraded* beyond the point where the DNA size is approximately the size of the STR loci being interrogated, analysis will be impossible. We believe all data suggests that there is indeed a window of opportunity as DNA sample are being concurrently degraded and damaged where repair (or partial repair) using our methods is possible.

In our first experiments we optimized a mixture of DNA polymerase I, dNTP's and T4 DNA ligase. This mixture is designed to 1) proofread 3' damaged bases, 2) fill in gaps, 3) nick translate through 5' damaged bases, and 4) seal nicks. When samples of each type of damage were treated with this mixture, the denatured molecular weight of the DNA increased. This was a good sign that at least some of the damage was being repaired, even with this minimal mixture that is lacking damage-removing nucleases.

For the next stage of the project we added individual repair enzymes to this optimized DNA polymerase/ligase mixture, and tested each on its ability to repair the different types of damaged DNA. Different amounts of each enzyme were used, and optimal levels were determined. One common observation was that for each enzyme there was indeed an optimal level, and that when too little was used, there was no increase in the number of loci observed, and when too much was used the number of loci obtained dropped even lower. Analysis of the DNA product showed a further decrease in the molecular weight of the DNA, indicating that each enzyme had a weak random endonuclease activity that was not targeted to damaged bases, but rather to DNA in general. This is a common observation, that nucleases can be forced to act indiscriminately when an excess of enzyme is present.

For the final stage of the project, enzyme mixtures or "blends" were evaluated that were predicted to repair many different types of damage. The goal of this section was to use the prior data obtained from the individual enzymes that each repair their own class of DNA damage to create a mixture that can be used to repair a wide variety of different types of damage. Interestingly, in developing the formulation of the enzyme mixture, the amount of each enzyme used needed to be decreased relative to the amount identified as optimal when only a single repair enzyme was added. Eventually, four repair enzymes to be added to the mixture of DNA polymerase and DNA ligase were finally chosen, which include;
1) *Fpg* (formamidopyrimidine [fapy]-DNA glycosylase, also known as 8-oxoguanine DNA glycosylase) which acts both as a N-glycosylase and an AP-lyase,
2) *Endonuclease IV* which can act on a variety of oxidative damage in DNA and is apurinic/apyrimidinic (AP) endonuclease that will hydrolyse intact AP sites in DNA,
3) *Endonuclease VIII* which acts as both an N-glycosylase and an AP-lyase, and
4) *T4-PDG* (pyrimidine dimer glycosylase) has both DNA glycosylase and AP-lyase activity that recognizes cis-syn-cyclobutane pyrimidine dimers caused by UV irradiation.

Once optimized, the final formulation was validated on the control damaged DNA using the exact protocols used in forensic laboratories. This validation confirmed that DNA samples yielding partial CODIS profiles can be at least partially repaired, depending on the extent of the damage and concurrent degradation, by including a simple 50 minute (total time) repair step after quantification and prior to the PCR reaction.

The developed repair method is as follows; a quantified DNA sample is added to the cocktail of nucleases, DNA polymerase and DNA ligase. This is incubated at 37 degrees for 30 minutes to allow for DNA repair. Then the sample is heated to 65 degrees for 20

minutes to completely inactivate all of the enzymes. After repair, 1 ng of product is removed directly to the PCR reactions for STR analysis. We have developed a 30 microliter repair reaction that can repair 15-30 ng of DNA, and 1-2 microliters of this reaction can be moved to the 25 microliter PCR analysis reaction. If DNA samples are limiting (<15 ng), we have determined that a 10 microliter reaction can be performed using 1 ng of input sample, and the entire reaction added to the 25 microliter PCR reaction.

While the work at GE Global Research was being conducted, there was also an effort underway at the North East Regional Forensic Institute (NERFI). This team's initial work was focused on generating human samples that had been exposed to various environmental conditions that are believed to result in damaged DNA. The goal of this effort was to develop a panel of samples that could be used to validate the DNA repair method. This validation would include the utilization of the exact methods used in a standard forensic laboratory, however with the addition of the DNA repair step. In this report we are including some of the methods used to create those samples, however this effort met with limited success.

The team at NERFI was also involved in testing various versions of the DNA repair method during method development. DNA was quantified using the Quantifiler™ Human DNA Quantification kit and evaluated using the AmpF*l*STR® Identifiler® PCR Amplification Kit carried out on the GeneAmp® PCR System 9700 with fragment separation and genotyping was performed on the Applied Biosystems Prism® 3130xl Genetic Analyzer and data review was performed using GeneMapper *ID* v.3.2 software. This was to ensure that the results obtained with the single PCR assay used by the GE team were consistent with the results that might be obtained using the exact methodology of a forensic laboratory. The methods section of this review contains two sections, one is a summary of the methods used by the GE team and the other is a summary of the methods used by the NERFI team.

Conclusions:

● As DNA samples are damaged by exposure to conditions that modify the chemical structure of the DNA, which can be repaired; there is concurrent degradation of the DNA, (degradation can not be repaired).

● There is likely a "window of opportunity for repair" during exposure of DNA to sub-optimal conditions when the DNA contains lesions that can be repaired and the molecular weight is large enough to still contain double stranded segments of DNA that encompass STR loci.

● Treatment of damaged DNA samples with a mixture of DNA polymerase and ligase can partially repair many samples.

● Addition of an optimized cocktail of enzymes including Fpg (formamidopyrimidine [fapy]-DNA glycosylase), E coli endonuclease IV, E coli endonuclease VIII and T4-PDG (pyrimidine dimer glycosylase) to the DNA polymerase/ligase mixture increases the ability of the mixture to repair DNA.

● Use of this repair method in the standard forensic lab workflow, as an additional step between quantification of the DNA and PCR, can increase the number of loci successfully scored.

● The repair method is compatible with the standard workflow used in forensic laboratories. The method has no adverse effect on un-damaged samples, which suggests that the implementation of this method as a standard practice in the processing of forensic samples could be beneficial.

● While the project did develop a repair reaction that can be used successfully, one potential drawback to the method involves the potential introduction of contaminating DNA, which could result in spurious allelic "drop-ins". Since none of the enzymes used in the repair method have been validated for use in a forensic setting, additional effort would be required by the suppliers of these enzymes to provide quality control assurance that each of the enzymes used was free of contaminating human DNA. Alternatively, a quality control step could be developed that would be used in the forensic laboratory that would be used each time the repair reaction is performed to confirm that in the absence of added sample the method did not introduce spurious DNA.

With the completion of this project, the team feels that this technology is now at a stage to allow for transition into manufacturing and production. Adequate proof of concept has been obtained demonstrating the efficacy of these methods. It is unlikely that the methodology could be implemented directly in the typical forensic laboratory without significant efforts to develop quality control procedures. While it is likely that a "home-brew" method could be prepared for use in research laboratories that might encounter degraded samples, use of the method in forensic laboratories would likely require commercialization of a "kit". Manufacturing and production of such a kit would allow for all of the components to be validated and the kit would be provided with quality control assurance.

In situations where DNA samples are know to be degraded and the intent is simply identification and not criminal determination, this method may have use immediately in the research laboratory setting. In cases such as these that do not involve the more strict requirements associated with criminal procedures, a "homebrew" method may be desirable. In this case, negative controls should be included to confirm that the reagents used are not contaminated fortuitously with human DNA. Additionally, reactions using undamaged positive control DNA should be performed prior to use on valuable samples to demonstrate that there has not been some fluctuation in the concentration of nuclease being added that might degrade the samples.

I. Introduction:

Statement of the problem:

DNA analysis performed at forensic laboratories today is confounded by DNA samples that have been exposed to uncontrolled environmental conditions. Typically, environmentally-damaged samples contain either degraded DNA fragments (double stranded breaks in the DNA) or contain somewhat intact DNA with single stranded nicks and DNA lesions. Damaged DNA contains nucleotide modifications in the DNA strands that are a block to the standard DNA replication machinery of the cell.[vii] As a result, nature has developed a plethora of cellular pathways designed to identify damaged DNA and eliminate it while preserving the competence of the genome. With the exception of recombinational repair, we have investigated the possibility of using commercially-available enzymes, which function in these pathways, to repair damaged DNA prior to amplification and analysis.

Statement of hypothesis or rationale for the research:

The main goal of the proposed program was to develop a method to repair DNA and determine its optimal method of use in forensic analysis. This method was designed to use an enzyme blend to fix environmentally-damaged samples that have DNA analysis issues. The method was designed to function after DNA isolation, but prior to DNA amplification. It is not anticipated that the method will be used on precious samples, but rather samples that are not limiting and have already yielded poor results using standard forensic methods, but that assumption may be incorrect if the method works reliably on all samples.

The underlying hypothesis at the start of the project, which did indeed seem to be correct, is that there is an "opportunity window for repair" during the damage/degradation of DNA. During this time the sample has not been completely degraded, yet contains base modifications which present a block to replication by DNA polymerase, and prevent the robust amplification of the loci needed for CODIS analysis. If samples are obtained that fall within this window, it should be possible to prepare an enzymatic solution capable of repairing the remaining DNA.

For DNA repair, three classes of enzyme can be used. The first is direct repair of the damage. This includes both DNA ligase, which seals single stranded nicks in the double stranded template, and photolyase, which catalyses the reversal of certain light induced damage. The second class are enzymes that identify base-damaged nucleotides and simply remove the base section of the nucleotide (glycosylase activity) leaving an intact sugar backbone. This product is referred to as an apurinic or apyrimidinic site (AP site) and is itself a block to replication. In vivo, AP sites are removed by AP endonucleases. In some cases, the glycosylase is associated with an AP endonuclease and both steps are accomplished by one enzyme. The product of this removal can be either nicked DNA with the AP site still associated with the strand, or a single base gap with the AP site eliminated, and in either case are still blocks to replication. Finally, there is nick translation. In this process, a DNA polymerase with the ability to both replicate DNA and simultaneously remove blocking single stranded DNA (5'-3' DNA polymerase and 5'-3' exonuclease activities) is utilized to move down a strand of DNA, essentially eliminating damaged bases in front of it while synthesizing an undamaged version. When finished, the enzyme dissociates, leaving a nick that can be sealed with ligase.

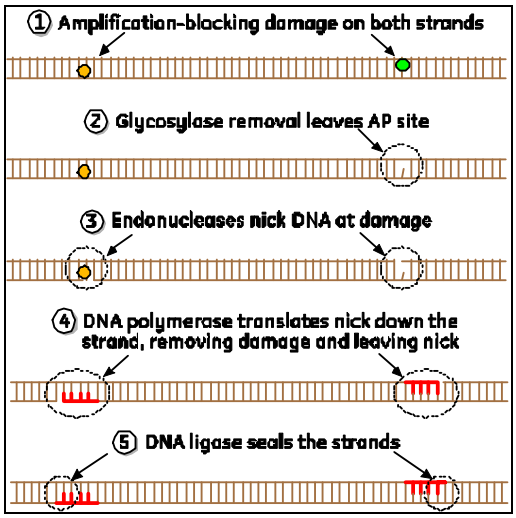


Figure 1- Repair Scheme

We have developed mixtures of enzymes (DNA ligase, various glycosylases and AP endonucleases, and DNA polymerase) that can be added to DNA containing any of a variety of damaged bases. After a simple incubation, during which the damage to the DNA is identified, removed, and DNA is sealed, the resulting sample can be moved to a variety of DNA analysis procedures including STR-based DNA fingerprinting.

Task list:

Task 1- Investigate the types of DNA damage commonly associated with environmentally damaged DNA.
To correctly develop a DNA repair method, it is essential to identify the major types of DNA damage that is typically associated with environmentally damaged DNA. In addition, knowing the prevalence of each type of damage and the frequency of each within samples is important to understand when assembling a method to repair these samples. The aim is to develop a formulation that can be used on most, if not all, samples to improve analysis results.

Task 2- Screen available DNA damage-targeted nucleases that will nick DNA containing the major classes of damaged DNA discovered.
The focus of the proposed program is not to "discover" new repair enzymes, rather to simply take advantage of the many commercially-available enzymes. Once common damage types are identified a collection of repair enzymes will be assembled. It is likely that many of these will be redundant, but this redundancy will be key to program progress, as assembling a mixture or blend of different enzymes can be difficult due to buffer restrictions. Having a variety of choices will facilitate the assembly of a single tube reaction.
Task 3- Develop a nuclease-based DNA polymerase/ligase repair method.
In this task we will use information from Tasks 1 and 2 to prepare and test enzyme blends that are predicted to either repair the DNA directly (in the case of nicked DNA and DNA dimer containing DNA) or to target the specific site of DNA damage for removal by the combined action of glycosylase/nuclease. The inclusion of a nick translation/ligation

system will be crucial to this mix for the removal of the damage (by the combined action of nuclease/DNA polymerase) and the subsequent closure of the DNA strand (ligase). Also included in this task will be both the synthesis of artificially-damaged templates and procurement of real world samples to test the different mix formulations. The genomic DNA sample will also be used to develop a model test system. Testing will include STR profiling analysis of the 13 CODIS loci.

Task 4- Validate the repair method of use in a real-life setting.
Once a formulation and method of use is determined, testing will be performed on genomic samples known to have analysis issues to validate the method and workflow using standard forensic laboratory workflows and instrumentation.

II. Methods

The following methods section is divided into two sections; methods used at GE-Global Research Center for methods development, and methods used at the Northeast Regional Forensic Institute (NERFI) for method validation. Some methods are close duplicates from the two sites.

GE-GRC methods-
GE-GRC:
1. Cell Culture of HT-29
HT-29: purchased from ATCC (#HTB-38)

1.1 Reagents:
The following media and buffers are required to culture, maintain the cell line:
~ McCoy's 5A (modified) with 1.5 mM L-glutamine adjusted to contain 2.2g/L sodium bicarbonate. (ATCC#30-2007, 500 ml)
~ Fetal bovine serum (Invitrogen #10099-141)
~ Trypsin-EDTA (Invitrogen, #25300-054)
~ PBS Dulbecco's w/o calcium, magnesium or sodium bicarbonate (Invitrogen #14190-094)

1.2 Growth-medium:
McCoy's 5A medium containing 1.5 mM L-glutamine and 2.2 g/L sodium bicarbonate which is adjusted to 10% fetal bovine serum (final concentration).

1.3 Cell thawing procedure:
~ Remove a cryo-vial from storage (liquid-Nitrogen)
~ Thaw the cells by holding the cryo-vial in a 37°C water bath for 1-2 min.
~ Remove the cryo-vial from the water bath and wipe with 70% ethanol.
~ Transfer the cells immediately under asceptic conditions to a T-75 flask containing 20 ml Growth-medium at 37°C.

1.4 Subculturing:
~ Remove and discard culture medium.
~ Briefly rinse the cell layer with 0.25% (W/V) Trypsin-0.53 mM EDTA solution to remove all traces of serum, which contains trypsin inhibitor.
~ Add 3 ml of Trypsin-EDTA solution to flask and contact cells entirely. Wait 3-10 min for cell detach.
~ Add 9 ml of complete growth-medium and aspirate cells by gently pipetting.
~ Transfer 4 ml of cell suspension to a new T-150 flask containing 20 ml Growth-medium (1:3 split ratio).

GE-GRC:
2. Harvest cells:
~ Remove the medium.
~ Wash the cells surface with 20 ml PBS, twice.

~ Add 5 ml Trypsin-EDTA and contact the cells entirely. Wait 10-15 min for cells detach.
~ Add 10 ml PBS and suspend the cells by gently pipetting.
~ Spin down the cells at 500rpm for 3 min and remove PBS.
~ Add another 10 ml PBS and wash cells by gently pipetting.
~ Spin down the cells at 500rpm for 5 min and remove PBS.
~ Store the cell pellets at –20°C.

GE-GRC:

3. Genomic DNA preparation (gDNA)

Use GE-Healthcare Kit (illustra tissue & cells genomicPrep Midi Flow Kit #28-9042-73). Follow manufacture instruction.

3.1 Reagents:

~ Lysis Solution: components
~ Load Buffer:
~ Elution Buffer 1:
~ **Proteinase K**, lyophilized power, store at 4°C. Reconstitute protein K in DNase-free water to give final concentration of 20 mg/ml.
~ **RNase A,** lyophilized power. Dissolve in DNase-free water to give final concentration of 20mg/ml (GE-Healthcare #406185)
~ **Elution Buffer 2 (10x TE)**: Prepare a 1:10 dilution of the Elution Buffer 2 to obtain a 1X TE Buffer solution.
~ Fast-Flow genomic 250 column (anion-exchange chromatography medium)
~ NAP-25 Desalting column
~ Millipore Steriflip filter

3.2 Cell lysis

~ Wash $1x10^7$ cells with 5 ml PBS twice. Suspend the cells in 5 ml 1x PBS and centrifuge at 2000xg for 10 min twice.
~ Re-suspend the cell pellet in 1 ml 1:10 diluted **Elution Buffer 2** (1xTE) by vortexing at highest speed for 30 sec for 1 min.
~ Add 4.5 ml of **Lysis Solution** and vortex for 15-30sec.
~ Add 50ul **Proteinase K** (20mg/ml) and vortex briefly.
~ Incubate at 60°C for 15-20 min and clear solution should be obtained.
~ Cool the tube in an ice bath for 2 min and add 20 ul of **RNase A** (20mg/ml). Incubate at 37C for 15 min.
~ Filter the sample using a Millipore Steriflip filter (0.22μm): wet the filter membrane with 1 ml of **Load Buffer**, filter the crude lysate and wash the membrane with 4 ml of **Load Buffer**.

3.2 Genomic DNA purification

Use Fast-Flow genomic 250 column.
Binding:
~ Transfer clear lysate containing genomic DNA to the column.

~ Place the column on a centrifuge tube and spin at 500rpm for 5 min by which time all the solution is passed through the resin.
~ Add 5 ml **Load Buffer** and spin at 500rpm for 2-5 min.
Elution:
~ Transfer the Fast-Flow genomic 250 column into a fresh centrifuge tube and add 2.5 ml of **Elution Buffer 1** directly onto the center of the column.
~ Spin the tube at 500rpm for 3 min and save the elution.

3.3 Desalting of purified genomic DNA
Use NAP-25 Desalting column.
~ Equilibrate the column with 25 ml of 1:10 diluted **Elution Buffer 2** (1x TE).
~ Transfer 2.5 ml of purified genomic DNA (from step 3.2) to the NAP-25 desalting column and allow the solution flow into the gel bed completely.
~ Transfer the column to a new centrifuge tube and elute the desalted product using 3.5 ml of 1:10 diluted **Elution Buffer 2** (1x TE Buffer)

GE-GRC:

4. Generation of oxidative damaged DNA samples:

materials

- Genomic DNA extracted from HT29 cell line with a final concentration of about 80-160ng/ul;
- 0.37M Iron Chloride ($FeCl_3$).
- 30% Hydrogen Peroxide (H_2O_2), ~8.8M
- NAP-5 Desalting Column (GE-Healthcare #17-0853-01)

- Prepare 100 ul of Fe-EDTA solution that is 18 mM in EDTA, and 9 mM in iron chloride by diluting 0.5 EDTA and 0.37 M $FeCl_3$ in water

- Prepare 1 ml 30 mM H_2O_2 solution on ice by adding 3.4 ul H_2O_2 stock to 1 ml water.

4.1 Damage DNA:

Make reaction mix in a tube, total reaction volume=30 ul, add H_2O_2 to start reaction.

Components	**Volume**
Genomic DNA(130ng/ ul)	1 ul
Fe-EDTA (9 mM-18 mM)	5 ul
dd H_2O	19 ul
30 mM H_2O_2	4 ul
Total Volume	**30 ul**

Incubate at 37°C for various times (20-100 min); desalting by using NAP-5 equilibrium with TE; elute in 1 ml TE.

- genomic DNA (extracted from HT-29 cell line) was damaged by Fenton reaction:
  - In each tube Mix: 2 ul genomic DNA (HT-29, 130ng/ul)
    5 ul Fe-EDTA (9 mM/18 mM)
    23 ul ddH2O
  - Add 4 ul 30 mM H_2O_2 to start reaction
  - Make 8 tubes of reaction
  - Incubate at 37°C for 80 min
  - Desalting by NAP-5 column
  - Eluted in 1 ml TE ([DNA]=2ng/ul)

4.2 Oxidative DNA sample preparation –

DNA oxidized by KMnO4

Materials:

- Genomic DNA extracted from HT29 cell line with a concentration of about 80-160ng/ul;
- 100 mM KMnO4
- TE buffer, pH8
- NAP-5 Desalting Column (GE-Healthcare #17-0853-01)

Damage DNA:

Make reaction mix in a tube, total reaction volume=30 ul, Add KmnO4 to start reaction.

Components	**Volume**
genomic DNA(130ng/ul)	2 ul
KmnO4 (100 mM)	3 ul
TE	25 ul
Total Volume	**30 ul**

Incubate at 37°C for various times (20-120 min); desalting by using NAP-5 equilibrium with TE; elute in 1 ml TE.

GE-GRC:
5 Acid/Heat damaged DNA (depurination) preparation

Rate of depurination of native deoxyribonucleic acid [viii]

Make 10xDepurination Buffer as indicated below, adjust pH=4.8:

Components	**Volume**
5M NaCl	0.2 ml
1M Sodium Phosphate (NaH_2PO_4)	0.1 ml
0.5M Sodium Citrate (Sigma #85,578-2)	0.2 ml
Water	9.5 ml
Total Volume	**10 ml**

1xDepurinationBuffer
0.1M NaCl
0.01M Sodium Phosphate
0.01M Sodium Citrate
pH=4.8

Damage DNA: make reaction in a tube with total volume=30 ul.

Components	**Volume**
Genomic DNA (130ng/ul)	1 ul
1xDepurinationBuffer	29
Total Volume	**30 ul**

Incubate at 70°C for 20-40hrs, desalting by using NAP-5 equilibrium with TE; elute in 1 ml TE.

GE-GRC:
6. Damage DNA by UVC:
100 ul of DNA (130ng/ul, extracted from HT-29 cell line) in a 1 cm path length quartz cuvette was exposed to UVC (245nm, ~4mw/cm^2) using a UV trans-illuminator for 1-15 min. Dilute to 2ng/ul.

GE-GRC:
7. MMS damaged DNA
Methyl Methanesulfonate (MMS) is an alkylating agent to DNA.
MMS damaged DNA preparation:

- Make 100 mM MMS in DMSA, seal the tube and store at 4°C.
- Alkylating the DNA (30 UL):

28 ul TE
1 ul HT-29 genomic DNA (130 ng/ul)
1 ul 100 mM MMS

Incubate at 37C for 2-60hrs.

GE-GRC:
8. double-damaged DNA

UVC exposure and Fenton reaction (oxidative)

- genomic DNA (extracted from HT-29 cell line) was damaged by Fenton reaction:
  - In each tube Mix: 2 ul genomic DNA (HT-29, 130ng/ul)
    5 ul Fe-EDTA (9 mM/18 mM)
    23 ul ddH2O
  - Add 4 ul 30 mM H2O2 to start reaction
  - Make 8 tubes of reaction
  - Incubate at 37C for 60 min
  - Desalting by NAP-5 column
  - Eluted in 1 ml TE ([DNA]=2ng/ ul)
    - Oxidative damaged DNA in a quartz curvet exposed to UVC for 3 min (245nm, ~4mw/cm^2)

GE-GRC:
9. DNA Repair:

Repair oxidative damaged DNA samples:
DNA repair reactions:
Repair enzymes:
- cv-PDG (Chlorella Virus Pyrimidine Glycosylase): Trevigen cat#4065-100-EB, 10U/ul:
- T4-PDG (T4 Endonuclease V or T4 Pyrimidine Dimer Glycosylase): NEB cat#M0308S, 10U/ul
- Endonuclease VIII, Trevigen #4060-01-EB, 10U/ul
- Endonuclease IV, NEB cat#M0304S, 10U/ul
- hOGG1, Trevigen, cat#4130-100-EB, 3U/ul
- Uracil DNA Glycosylase (UDG): NEB cat#M0280S, 2U/ul.
- Endonuclease III, Trevigen #4045-01K-EB, 10U/ul

Notes:
- Dilute multiple repair enzymes in a single tube with 1X Repair Buffer when needed.

Repair:

Components	**Volume (ul)**	**Final**
10X Repair Buffer	3	1x
10 mM ATP	3	1 mM
1 mM dNTP	3	100uM
1mg/ml BSA	3	0.1mg/ml
E.coli Pol I (10U/ul)	1	10U
T4 DNA Ligase (400U/ul)	1	400U
Fe3+/H2O2-80 min DNA (2ng/ul)	15	30ng or ~ 1ng/ul
enzyme as indicated	1	indicated
Total Volume	**30 ul**	

Incubate at 37°C/30 min, 65°C/20 min.

Repair Acid/Heat damaged DNA (depurination) preparation

- DNA sample: treated with Depurination Buffer for 40hrs at 70°C, desalting, final [DNA] = 2ng/ul**.**
- Endonuclease III: Trevigen, cat#4045-10K-EB, 10U/ul

Endonuclease III releases damaged bases induced by UV, ionizing radiation, osmium tetroxide, or acid. It is a DNA glycosylase with an associated AP lyase activity and contains an iron-sulfur group which helps to maintain its three dimensional conformation. Endonuclease III cleaves as a DNA lyase at abasic sites by b-elimination. This results from a b-elimination reaction, producing a single nucleotide gap in the DNA.

Components	**Volume (ul)**	**Final**
10X RepairBuffer	3	1x
10 mM ATP	3	1 mM
1 mM dNTP	3	100uM
1mg/ml BSA	3	0.1mg/ml
E.coli Pol I (10U/ul)	1	10U
T4 DNA Ligase (400U/ul)	1	400U
DNA (2ng/ul)	15	30ng or 1ng/ul
enzyme as indicated	1	indicated
Total Volume	**30 ul**	

Incubate at 37°C/30 min, 65°C/20 min.

Repair UVC Damaged DNA:

Repair enzymes:

- cv-PDG (Chlorella Virus Pyrimidine Glycosylase): Trevigen cat#4065-100-EB, 10U/ul:
- T4-PDG (T4 Endonuclease V or T4 Pyrimidine Dimer Glycosylase): NEB cat#M0308S, 10U/ul
- Endonuclease VIII, Trevigen #4060-01-EB, 10U/ul
- Endonuclease IV, NEB cat#M0304S, 10U/ul
- hOGG1, Trevigen, cat#4130-100-EB, 3U/ul
- Uracil DNA Glycosylase (UDG): NEB cat#M0280S, 2U/ul.
- Endonuclease III, Trevigen #4045-01K-EB, 10U/ul

Notes:

- Dilute multiple repair enzymes in a single tube with 1X Repair Buffer when needed.

Repair :

Components	**Volume (ul)**	**Final**
10X RepairBuffer	3	1x
10 mM ATP	3	1 mM
1 mM dNTP	3	100uM
1mg/ml BSA	3	0.1mg/ml
E.coli Pol I (10U/ul)	1	10U
T4 DNA Ligase (400U/ul)	1	400U
UVC-5 min-nov13 DNA (2ng/ul)	15	30ng or ~ 1ng/ul
enzyme as indicated	1	as indicated
Total Volume	**30 ul**	

Incubate at 37°C/30 min, 65°C/20 min.

Repair double damaged DNA – UVC exposure and Fenton reaction (oxidative)

Repair:

Components	**Volume (ul)**	**Final**
10X RepairBuffer	3	1x
10 mM ATP	3	1 mM
1 mM dNTP	3	100uM
1mg/ml BSA	3	0.1mg/ml
E.coli Pol I (10U/ul)	1	10U
T4 DNA Ligase (400U/ul)	1	400U
Fe3+/H2O2-UVC3' DNA (2ng/ul)	15	28ng or ~1ng/ul
enzyme as indicated	1	
Total Volume	**30 ul**	

Incubate at 37°C/30 min, 65°C/20 min.

10. Analysis of DNA repair by PCR

PCR repaired DNA:

Components	**Volumes**
Repaired DNA (1ng/ul)	2 ul
P-Set-14 (2.5uM)	1.5 ul
ddH_2O	21.5 ul
ReadyToGo-PCR beads (GE-Healthcare#27-9558-01)	1
Final Volume	**25 ul**

PCR parameters:
95°C, 5 min
[95°C/30s, 58.5°C/30s, 72°C/90s] cycles=**38**
72°C, 10 min

All PCR samples were mixed with loading buffer, run on denaturing polyacrylamide gels, stained with SYBR gold (Molecular Probes) and scanned using a Typhoon Variable Mode Gel Imager (GE Healthcare). PCR reactions were performed on the DNA samples using individual primer sets designed to amplify the 13 separate CODIS STR loci, plus a 14^{th} loci, non-STR control. For every DNA sample these 14 PCR reactions were performed, and the resulting material was analyzed by denaturing TBE-urea PAGE.

Lanes on gel		
1	**MW**	**marker**
	STR loci	**Primer ID**
2	CSF1PO	5′-d[CCG GAG GTA AAG GTG TCT TAA AGT]-3′. 5′-d[ATT TCC TGT GTC AGA CCC TGT T]-3′
3	D3S1358	5′-d[ACT GCA GTC CAA TCT GGG T]-3′ 5′-d[ATG AAA TCA ACA GAG GCT TGC]-3′
4	D5S818	5′-d[GGT GAT TTT CCT CTT TGG TAT CC]-3′ 5′-d[AGC CAC AGT TTA CAA CAT TTG TAT CT]-3′
5	D7S820	5′-d[ATG TTG GTC AGG CTG ACT ATG]-3′ 5′-d[GAT TCC ACA TTT ATC CTC ATT GAC]-3′
6	D8S1179	5′-d[GCA ACT TAT ATG TAT TTT TGT ATT TCA TG]-3′. 5′-d[ACC AAA TTG TGT TCA TGA GTA TAG TTT C]-3′
7	D13S317	5′-d[ACA GAA GTC TGG GAT GTG GAG GA]-3' 5′-d[GGC AGC CCA AAA AGA CAG A]-3′
8	D16S539	5′-d[GGG GGT CTA AGA GCT TGT AAA AAG]-3′ 5′-d[GTT TGT GTG TGC ATC TGT AAG CAT GTA TC]-3′
9	D18S51	5′-d[TTC TTG AGC CCA GAA GGT TA]-3′. 5′-d[CTA CCA GCA ACA ACA CAA ATA AAC]-3′
10	D21S11	5′-d[ATA TGT GAG TCA ATT CCC CAA G]-3′ 5′-d[TGT ATT AGT CAA TGT TCT CCA GAG AC]-3′
11	FGA	5′-d[GGC TGC AGG GCA TAA CAT TA]-3′ 5′-d[TT CTA TGA CTT TGC GCT TCA GGA]-3′
12	TH01	5′-d[GTG ATT CCC ATT GGC CTG TTC]-3′. 5′-d[TCC TGT GGG CTG AAA AGC TC]-3′
13	TPOX	5′-d[GCA CAG AAC AGG CAC TTA GG]-3′ 5′-d[CGC TCA AAC GTG AGG TTG]-3′
14	vWR	5′-d[GCC CTA GTG GAT GAT AAG AAT AAT CAG TAT GTG]-3′ 5′-d[GGA CAG ATG ATA AAT ACA TAG GAT GGA TGG]-3′
15	AMEL control	5′-d[CCC TGG GCT CTG TAA AGA A]-3′ 5′-d[ATC AGA GCT TAA ACT GGG AAG CTG]-3′

Table 1: Primer sets used for STR loci amplification[ix]

GE-GRC:

11. Repair Damaged DNA, final formulation:

Reagents:

10x RepairBuffer: 100 mM Tris-HCl pH=7.9, 100 mM $MgCl_2$, 500 mM NaCl, 10 mM dithiothreitol
10 mM ATP (Ambion #8110G)
BSA (1mg/ml, diluted from 10 mg/ml NEB#B9001S))
2mM dNTP (GE-Healthcare #28-4065-62)
E. coli DNA polymerase I (NEB #M0209S, 10 U/ul)
T4 DNA Ligase (NEB#M0202S, 400 U/ul)
Endonuclease IV (NEB # M0304S, 10 U/ul) –dilution in 1x RepairBuffer.
Endonuclease VIII (Trevigen #4060-01-EB, 10 U/ul) –dilution in 1x RepairBuffer.
Fpg / E. coli 8-oxoguanine DNA glycosylase (NEB #M0240S, 8 U/ul) – dilution in 1x RepairBuffer.
T4 Endonuclease V (T4-PDG or T4 Pyrimidine Dimer Glycosylase) (NEB #0308S, 10U/ul) – dilution in 1x RepairBuffer.
Endo IV, endo VIII, T4-PDG and Fpg can be combined into an appropriate dilute mixture in 1x RepairBuffer immediately prior to addition to the repair reaction. All enzyme solutions must be maintained on ice, and mixtures should be prepared immediately prior to use.

Repair Reaction:

Components	**Final**
Tris-HCl, pH=7.9	10 mM
$MgCl_2$	10 mM
NaCl	50 mM
DTT	1 mM
ATP	1 mM
BSA	0.1 mg/ml
dNTP	0.2 mM
E. coli DNA Polymerase I	3.3 units
T4 DNA Ligase	133 units
T4-PDG	33 milliunits
Endo IV	33 milliunits
Endo VIII	33 milliunits
Fpg	3 milliunits
Damaged DNA	1-10 ng
Total volume	10 ul

Incubate at 37°C for 30 min,
Heat inactivate mixture, 65°C for 20 min.
Remove 1 ng (1-10 microliters) directly to PCR reaction for STR analysis.

Methods used at NERFI-

The general procedures for DNA extraction, STR amplification, and capillary electrophoresis (CE) which were performed at NERFI are described in the following section and were used for all sample types. Specific details regarding sample volumes or concentrations, sample injection times, or modifications are noted within the sections below for each sample type. Each extraction included a reagent blank. Each STR amplification included the extraction reagent blank, a positive control (AmpFℓSTR® Control DNA 9947A, Applied Biosystems, Foster City, CA), and a negative control consisting of water or TE buffer. Unless otherwise noted, the positive and negative controls performed as expected.

NERFI

1. DNA Extraction

DNA was isolated following an organic extraction procedure. Samples were incubated in extraction buffer (EB) (10 mM Tris pH 7.5, 10 mM EDTA, 50 mM NaCl, 2% SDS, 300 µg/mL proteinase K) with 20 µL 1M DTT for 2 hours at 56°C. Bloodstains and swab tips were then placed in Spin-Ease tubes (Gibco-BRL, Grand Island, NY) and centrifuged to collect all the liquid in the EB. The samples were then subjected to phenol:chloroform:isoamyl alcohol (50:48:2) extraction with a butanol wash, followed by several washes in TE^{-4} buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 8.0) in a MicroCon® 100 Centrifugal Filter (Millipore, Bedford, MA) to concentrate the DNA. The blood DNA was routinely resuspended in ~60 µL of TE and the saliva DNA in ~25 µL of DNA.

2. DNA Quantification

DNA quantification was not performed on the blood or saliva. With regard to the bloodstains, DNA quantity was based on the estimate that approximately 250 ng of DNA is contained in a $3mm^2$ cutting from a dried bloodstain. The decision to omit quantification on the saliva was based on the following: Earlier experiments in the lab in which DNA quantity in saliva swabs was determined using the Quantifiler™ Human DNA Quantification Kit (Applied Biosystems, Foster City, CA) did not necessarily correlate with successful STR amplification. When DNA is degraded or damaged, the larger STR loci—those in the 300–500 bp range—are usually the first to fail in amplification ([x], [xi]). Because the quantification assay targets a 62 b sequence, it could overestimate the quantity of intact DNA present in a specimen. Since our saliva samples were limiting, we chose to omit the quantification step. With regard to the dried bloodstains, we relied on estimated DNA quantities for these initial experiments with the intent to perform quantification on all sample types later.

3. PCR Amplification and Capillary Electrophoresis

STR amplification of the 13 CODIS core loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, vWA, TPOX, D18S51, D5S818, and FGA), the sex-typing locus amelogenin, and two additional loci (D2S1338 and D19S433)

was performed using the AmpF*l*STR® Identifiler® PCR Amplification Kit (Applied Biosystems, Foster City, CA). Preparation of the PCR master mix and amplification was performed according to the manufacturer's instructions. The reaction volume was 25 µL and amplification was carried out on the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). PCR conditions were as follows: initial incubation at 95°C for 11 min, then 28 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for min, a final incubation at 60°C for 60 min, and a hold at 10°C until storage at 2-8°C.

To prepare the samples for fragment analysis, 2 µL of PCR product was added to 24.5 µL Hi-Di™ Formamide (Applied Biosystems, Foster City, CA) and 0.5 µL GeneScan™ 500 LIZ® Size Standard (Applied Biosystems, Foster City, CA). Fragment separation and genotyping was performed on the Applied Biosystems Prism® 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) using POP-4 Polymer. Specific sample injection times are noted throughout the text, but were typically 5 and/or 20 s. Data review was performed using GeneMapper *ID* v.3.2 software (Applied Biosystems, Foster City, CA) with a 50 RFU threshold.

NERFI
4. Cell Line DNA

Sample Preparation

In order to have a plentiful supply of DNA to damage and repair, GE-GRC chose to develop repair methods using a cell line derived from a human (female) adenocarcinoma—HT29 (American Type Culture Collection, Manassas, VA). Cell culture, extraction, and DNA quantification were carried out at GE-GRC and samples were transferred to NERFI

NERFI
5. Artificially Induced Damage

In an effort to induce UV damage in the human DNA, 60 µL of HT29 DNA (130 ng/µL) was added to a quartz cuvette, and exposed to UVC radiation (254 nm) by placing it on a UV trans-illuminator. After 5 min, 20 µL were removed. After an additional 2 min, another 20 µL was removed. HT29 DNA was also subjected to oxidative damage[xii] by mixing HT29 DNA with Fe-EDTA, distilled water, and H_2O_2 and incubating at 37°C for 60 min. Iron, a PCR inhibitor, was subsequently removed by desalting (NAP-5 Column, GE Healthcare, Piscataway, NJ).

NERFI
6. DNA Repair

Damaged HT29 cell line DNA (UVC and oxidative) was repaired according to the following protocol. Fifteen µL of damaged HT29 DNA (final concentration of 1 ng/µL) was added to a reaction mix containing 3 µL 10X NEBuffer (New England BioLabs, Ipswich, MA), 3 µL 10 mM ATP, 3 µL 1 mM dNTP, 1 µL E. *Coli* polymerase I (10 U/µL), 1 µL T4 DNA ligase (400 U/µL), 3 µL BSA (1 mg/ml), and 1 µL of a specific repair enzyme, for a total reaction volume of 30 µL. The specific enzyme used to repair the UVC-damaged DNA was chlorella virus pyrimidine dimer glycosylase (cv-PDG)

(Trevigen, Gaithersburg, MD). The enzyme uracil DNA glycosylase (UDG) (New England BioLabs, Ipswich, MA) was used to repair the DNA damaged by oxidation. The reaction mix was incubated at 37°C for 30 min. The reaction was stopped by incubating at 65°C for 20 minutes.

According to their respective manufacturers, cv-PDG is a DNA glycosylase, with AP lyase activity, that targets cyclobutane pyrimidine dimers caused by UVR; and UDG catalyzes the removal of uracil from DNA.

7. PCR Amplification and Capillary Electrophoresis

To assess the performance of HT29 DNA damaged and repaired at GE, in the multiplex PCR and capillary electrophoresis platform in our laboratory, the HT29 DNA was amplified using the AmpF*ℓ*STR® Identifiler® PCR Amplification Kit at 1 and 10 ng. Capillary electrophoresis was performed with 5 and 20 s sample injections. In a separate experiment to evaluate whether the enzyme reaction components affected the STR amplification, damaged and repaired HT29 DNA was subjected to an additional organic extraction to remove the repair reaction components. Following re-extraction, the damaged and repaired HT29 DNA was amplified using 1 and 2 ng. CE was then carried out with a 20 s sample injection. Finally, an STR amplification of the damaged and repaired HT29 DNA was performed using 10 ng with a subsequent 20 s sample injection. Increasing the quantity of DNA in the PCR reaction and using a 20 s CE injection were done in an effort to bring up more alleles in the profiles.

NERFI

8. Blood

Simulated Forensic Sample Preparation

EDTA-preserved whole human blood samples from five subjects (6707A, 62107A, 62107B, 62107C, and 62107D) were obtained from U.S. Biologicals (Swampscott, MA). Each bloodstain was prepared by spotting 25 µL of whole blood from each of the five subjects onto autoclaved 100% cotton cloth. Several bloodstains were prepared for each subject. The stains were air-dried and stored in paper envelopes at 4°C until use. Samples from each of the five subjects were extracted and amplified upon receipt to develop normal control profiles.

Artificially Induced Damage

Although UVC is a strong inducer of DNA damage, most of the UVC from the sun is absorbed by the atmosphere ([xiii]). Under natural conditions there are insufficient levels of UVC to cause much damage in crime scene evidence. Most of the UV radiation that passes through the atmosphere is comprised of UVA and UVB. In an attempt to mimic the damage caused by natural sunlight, but in a more timely fashion that might occur naturally, the dried bloodstains were incubated on two occasions in a laboratory weather chamber, the Ci4000 Weather-Ometer® (Atlas Material Testing Technology, Chicago, IL) (hereinafter "weather chamber"). The weather chamber is described as having the sunlight intensity of Miami Beach at noon during spring break. On the first occasion, dried bloodstains from subjects 6707A, 62107A, 62107B, 62107C, and 62107D

were exposed in the weather chamber for 1, 20, 50, 105, and 165 min (2 bloodstains per subject for each time point). The relevant instrument settings on the chamber were as follows: chamber temperature, 35°C; relative humidity, 30%; and irradiance, 0.75W. On the second occasion, previously exposed (165 min) bloodstains from each subject were exposed for an additional 24 h in the same weather chamber under the same machine conditions. After exposure in the weather chamber on both occasions, the samples were stored in paper envelopes at 2-8°C until DNA extraction.

Naturally Induced Damage

Dried bloodstains from all 5 subjects were placed on the sill of a window exposed to uninterrupted daylight. Cuttings were removed from all 5 subjects at 4 and 12 and 20 weeks and held in paper envelopes at 2-8°C until DNA extraction.

DNA Extraction

An approximately 3 mm^2 section was cut from each bloodstain. For the first weather chamber experiment, only cuttings from subject 6707A were extracted. For the second weather chamber experiment, one cutting from each of the 5 subjects was taken for extraction. For the 4-week window light experiment, one cutting from each of subjects 6707A, 62107B, and 62107D was extracted.

PCR Amplification and Capillary Electrophoresis

Bloodstain DNA extracts from all experiments were amplified using an estimated 1 ng of template DNA.

NERFI

9. Saliva

Simulated Forensic Sample Preparation

Aluminum beverage cans were cleaned with 10% bleach to remove any contaminating DNA and then rinsed several times with sterile water. Three subjects (one male and two females) then drank from several cleaned aluminum beverage cans each. The cans were then stored in the dark at ambient until use in damaging experiments or immediately exposed to window light. Beverage cans S1, S2, and S3 (from one female donor, Subject X) were held in the dark to be used as negative controls for damage.

Artificially Induced Damage

The beverage cans with saliva deposits were incubated in the laboratory weather chamber contemporaneously with the dried bloodstains. On the first occasion, the mouth areas containing saliva deposits were cut from 10 beverage cans used by one female donor (Subject X) and were exposed in the weather chamber as follows. Sample SS1 for 1 min, SS2 for 2 min, SS3 for 20 min, SS4 for 20 min, SS5 for 50 min, SS6 for 50 min, SS7 for 105 min, SS8 for 105 min, SS9 for 2 min, and SS10 for 165 min. On the second occasion, the mouth areas from 2 cans (one from each of two female donors—Subjects A and X) were exposed in the instrument for 24 h. These cans had not been previously exposed in the instrument. After exposure on both occasions, the samples were stored in paper envelopes at 2-8°C (or ambient) until DNA extraction.

Naturally Induced Damage

Eight beverage cans with saliva deposits from one male donor (Subject Z) and 8 from one female donor (Subject A) were placed in the sill of a window exposed to uninterrupted daylight for 4 weeks. The mouth areas of the cans were immediately swabbed and the swabs were stored in paper envelopes at 2-8°C until DNA extraction.

DNA Extraction

Saliva was collected from the mouth area of each beverage can using sterile cotton swabs.

PCR Amplification and Capillary Electrophoresis

STR amplification of all damaged saliva samples was performed using 10 µL sample volume. Saliva control samples S1, S2, and S3 were amplified using both 10 and 1 µL volumes. The CE sample injection time for the saliva controls was 20 s for the 10 µL amplification volume and 5 s for the 1 µL amplification volume. CE of the saliva samples exposed in the first weather chamber experiment was carried out with 5 and 20 s injections. The saliva samples exposed in the second weather chamber experiment were subjected to CE with a 20 s injection. CE of the saliva samples exposed for 4 weeks to daylight was carried out with a 5 s injection.

NERFI

10. Final formulation testing

Simulated Forensic Sample Preparation

Control undamaged USB 62107A chromosomal DNA, 80 minute Fe/H_2O_2 oxidative damaged DNA (see figure 2 and figure 17), and 28 hr heat/acid damaged DNA (see figure 4 and figure 16) concentration was determined using the Quantifiler Human DNA Quantification Kit (Applied Biosystems, Foster City, CA). A 20 ul repair reaction containing 2 ng (as determined by Quantifiler) of undamaged or damaged DNA was performed. Because the quantification assay targets a 62 bp sequence, it could overestimate the quantity of intact DNA present in a specimen that can be sized using the Identifiler kit as the sizes of these amplicons can be greater than 300 bp. The repair reaction conditions were exactly as described in section 10: "final formulation" of the GE-GRC methods. After repair and enzyme inactivation by heating to 65 degrees for 20

minutes, 10 ul of repaired DNA (1 ng, as determined prior to repair) or 1 ng of non-repaired DNA was added the AmpF*l*STR Identifiler PCR Amplification Kit (Applied Biosystems, Foster City, CA). Preparation of the PCR master mix and amplification was performed according to the manufacturer's instructions. The reaction volume was 25 µL and amplification was carried out on the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). PCR conditions were as follows: initial incubation at 95°C for 11 min, then 28 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for min, a final incubation at 60°C for 60 min, and a hold at 10°C until storage at 2-8°C.

To prepare the samples for fragment analysis, 2 µL of PCR product was added to 24.5 µL Hi-Di™ Formamide (Applied Biosystems, Foster City, CA) and 0.5 µL GeneScan™ 500 LIZ® Size Standard (Applied Biosystems, Foster City, CA). Fragment separation and genotyping was performed on the Applied Biosystems Prism® 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) using POP-4 Polymer. Specific sample injection times are noted throughout the text, but were typically between 5 and 20 s. Data review was performed using GeneMapper *ID* v.3.2 software (Applied Biosystems, Foster City, CA) with a 50 RFU threshold.

Table 2. A summary of the enzymes and their target and activities evaluated in this study

Enzyme	Lesion	Activity
E. coli DNA polymerase I	▪ 3' modified DNA end ▪ 3' Single stranded terminal mismatch ▪ Gaps	3'-5' exonuclease 5'-3' exonuclease 3'-5' DNA polymerase
T4 DNA ligase	▪ Nicked DNA	T4 DNA ligase seals adjacent termini on nicked double stranded DNA
E. coli 8-oxoguanine DNA glycosylase (Fpg)	▪ 7, 8-dihydro-8-oxoguanine (8-oxoguanine) ▪ 8-oxoadenine ▪ Fpy-guanine ▪ Methy-fapy-guanine ▪ Fapy-adenine ▪ Aflatoxin B1-fapy-guanine ▪ 5-hydroxy-cytosine ▪ 5-hydroxy-uracil	The N-glycosylase activity releases damaged purines from double stranded DNA, generating an AP site. The AP-lyase activity cleaves both 3′ and 5′ to the AP site thereby removing the AP site and leaving a 1 base gap.
Human hOGG1 (α isoform) 8-oxoguanine DNA glycosylase	▪ 7, 8-dihydro-8-oxoguanine (8-oxoguanine) when base paired with cytosine ▪ 8-oxoadenine when base paired with cytosine ▪ Foramidopyrimidine (fapy)-guanine ▪ Methy-fapy-guanine	The N-glycosylase activity releases damaged purines from double stranded DNA, generating an AP site. The AP-lyase activity cleaves 3′ to the AP site leaving a 5′ phosphate and a 3′-phospho-α, β-unsaturated aldehyde.
Mouse 3-Methyladenine DNA Glycosylase Type II	▪ 3-methyladenine ▪ 3-methylguanine ▪ 7-methylguanine ▪ Hypoxanthine	The N-glycosylase activity releases damaged purines from double stranded DNA, generating an AP site.
Chlorella Virus Pyrimidine Dimer Glycosylase (cvPDG)	▪ Cis-syn and trans-syn isomers of cyclobutane pyrimidine dimers	The N-glycosylase activity releases damaged bases from double stranded DNA, generating an AP site. The associated AP lyase activity nicks the duplex DNA to produce single-strand gaps.
Human apurinic/apyrimidinic endonuclease (APE 1)	▪ Urea ▪ 5, 6 dihydroxythymine, thymine glycol ▪ 5-hydroxy-5 methylhydanton ▪ Uracil glycol ▪ 6-hydroxy-5, 6-dihdrothimine ▪ Methyltartronylurea	Nicks backbone immediately 5′ to an AP site

Table 2. A summary of the enzymes and their target and activities evaluated in this study

Enzyme	Lesion	Activity
E coli Endonuclease III (Nth)	▪ Urea ▪ 5, 6 dihydroxythymine ▪ Thymine glycol ▪ 5-hydroxy-5 methylhydanton ▪ Uracil glycol ▪ 6-hydroxy-5, 6-dihdrothimine ▪ Methyltartronylurea	AP-lyase activity of the enzyme cleaves 3′ to the AP site leaving a 5′ phosphate and a 3′ ring opened sugar.
Endonuclease IV	▪ Apurinic/apyrimidinic site	AP sites are cleaved at the first phosphodiester bond that is 5′ to the lesion leaving a hydroxyl group at the 3′ terminus and a deoxyribose 5′-phosphate at the 5′ terminus
E. coli Endonuclease V	▪ Deoxyinosine ▪ Abasic sites ▪ Urea ▪ Base mismatches ▪ Insertion/deletion mismatches ▪ Hairpin or unpaired loops ▪ Flaps ▪ Pseudo-Y structures	Endonuclease V cleaves the second phosphodiester bond 3′ to the deoxyinosine
E. coli Endonuclease VIII	▪ Urea ▪ 5, 6- dihydroxythymine ▪ Thymine glycol ▪ 5-hydroxy-5- methylhydanton ▪ Uracil glycol ▪ 6-hydroxy-5, 6-dihydrothymine ▪ Methyltartronylurea	The N-glycosylase activity releases damaged pyrimidines from double-stranded DNA, generating an AP site. The AP-lyase activity cleaves 3′ and 5′ to the AP site leaving a 5′ phosphate and a 3′ phosphate.
T4 Endonuclease V (T4 PDG)	▪ Cis-syn isomer of cyclobutane pyrimidine dimer	The N-glycosylase activity releases damaged bases from double stranded DNA, generating an AP site. The associated AP lyase activity nicks the duplex DNA to produce single-strand gaps.
E. coli Exonuclease III	▪ RNase H (RNA:DNA duplex) ▪ 3′-phosphatase ▪ AP-endonuclease activities	3’-5’ dsDNA exonuclease. The preferred substrates are blunt or recessed 3′-termini, although the enzyme also acts at nicks in duplex DNA to produce single-strand gaps. Nicks DNA containing RNA or AP sites.
E. coli Photolyase	▪ Cyclobutane pyrimidine dimers	Light energy drives electron transport from a catalytic chromophore, reduced FADH, to the pyrimidine dimer, leading to its photolysis
Uracil DNA Glycosylase (UDG)	▪ uracil-containing DNA	Catalyses the release of free uracil from uracil-containing DNA. UDG efficiently hydrolyzes uracil from single-stranded or double-stranded DNA

III. Results

Work at GE-GRC:

In the first part of the project the team needed to develop a model system for an evaluation of any DNA repair methodology. In order to obtain a large quantity of human chromosomal DNA the tissue cell line was obtained and high quality DNA was purified from the cells. Aliquots from this sample were then used in reactions designed to damaged DNA. The idea was to find the treatment that would yield DNA that gave partial profiles when tested in a PCR based STR sizing protocol. For ease of use we simply used one nanogram of DNA in each of the PCR reactions as determined by OD260 measurements of the material prior to damage. We did not re-quantify the DNA after damage, we just used what would have been one nanogram of the starting material. Since we know that partial profiles are obtained in forensic labs regularly, we just wanted to make a model DNA that would also give a partial profile using our 14-loci single-plex PCR method.

We first generated DNA that was damaged by Fenton reaction. In this reaction, hydrogen peroxide and iron are mixed with the DNA resulting in oxidative damage. The resulting DNA from time course of Fenton reaction is shown in figure 2. It is clear from this experiment that in addition to DNA damage which results in modified bases, the DNA sample is also been degraded. It is essential for this method that the DNA degradation does not outpace the DNA damage, as DNA degradation can not be repaired. Each time point in this reaction was saved and tested in the PCR based STR sizing test, and the time point that gave a partial profile was used to test DNA repair reactions.

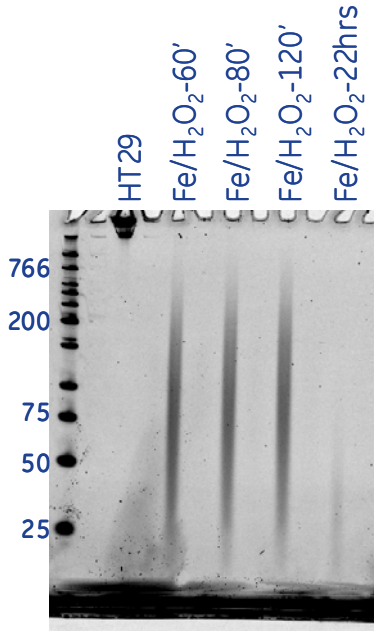


Figure 2: Time-course of oxidative damage to DNA. Note the decrease in molecular weight as damage proceeds.

This process of generating damaged DNA samples was repeated using a potassium permanganate reaction, which also creates oxidative damage, and with a reaction that heats the DNA in acidic solution, which causes base modifications. In our first heat acid treatment the molecular weight of the DNA was extremely low (figure 3), the so we repeated that experiment with shorter time points (figure 4). It is unclear why the potassium permanganate treated samples did not show up on this gel as we were able to obtain a damaged-DNA profile from these. It is possible that the damage to the DNA prevented the intercalating dye from binding efficiently, or that the molecular weight of the DNA was too low to be resolved on this gel system.

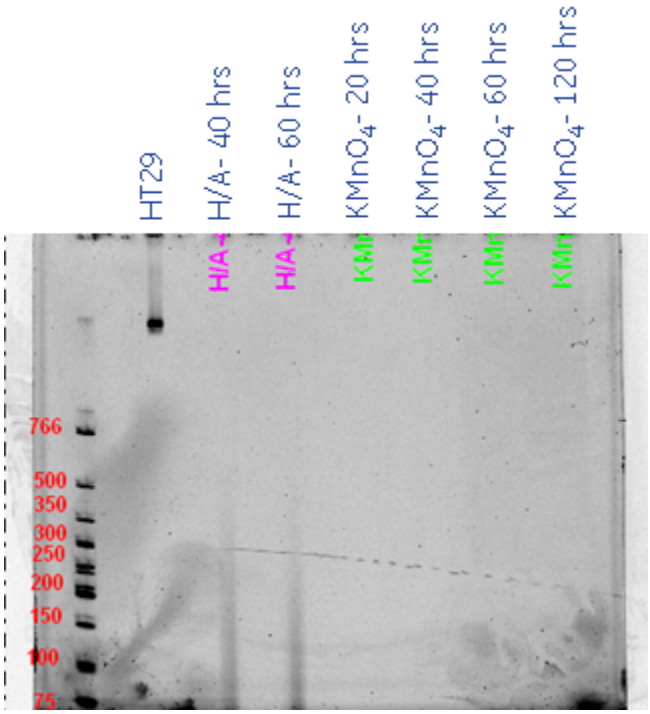


Figure 3: Time course of oxidative damage to DNA. Unable to visualize the KMnO4 damaged DNA of on TBE-Urea gel. The amount of DNA must be too low to be able to see on the gel.

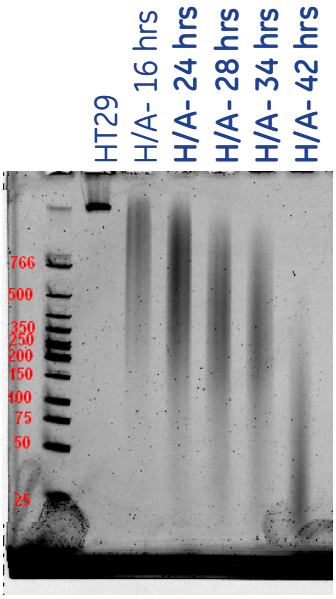


Figure 4. Time course of heat/acid treatment to DNA. Note decrease in molecular weight as damage proceeds.

A DNA damaging method was also developed for UV damage. While UV damage involving cyclobutane dimers is known to occur on DNA samples in an aqueous environment, the rate of this damage is slowed significantly in lyophilized samples. Since it is impossible to predict whether or not forensic samples will be damaged by sunlight, we felt it important to develop a repair reaction that could address UV damaged samples. In this particular case we were using a UVC light source, however later in the project an aging chamber was utilized that can expose samples to the same UV profile as natural sunlight. In figure 5 the DNA from a UV damaging experiment has been sized.

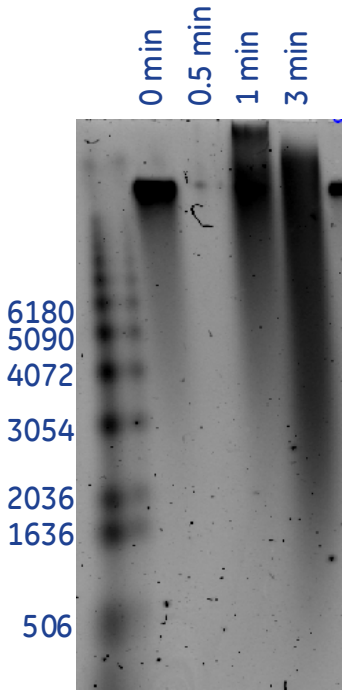


Figure 5: Time course of UV-C damage to DNA. Note, DNA samples are in solution, where DNA damage is known to occur. We would not predict this result if the sample has been dried DNA as damage proceeds much more slowly. Note also, decrease in molecular weight as damage proceeds.

We also damaged DNA with methyl methanesulfonate, which is a known DNA alkylating agent. While he decrease in molecular weight was observed (figure 6), we were not able to generate samples that would only give a partial profile in our STR analysis protocol. The un-repaired samples gave either a complete profile or no profile at all.

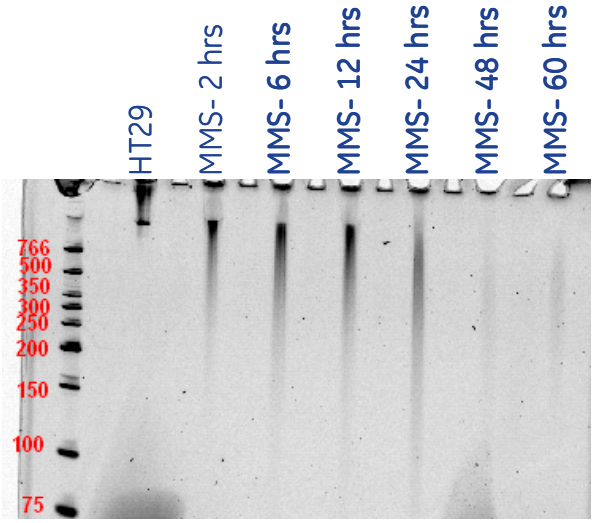


Figure 6: Alkylation damage time course. MMS-60hrs-damaged DNA still showed full STR loci without using repair enzyme, indicating that the nicking and degradation of DNA by MMS was a slow process.

Now that we had DNA samples which gave us partial profiles in our STR analysis protocol, we started to develop the repair protocol. The first thing that we did was to develop a nick translation reaction using E. coli DNA polymerase I. This enzyme has three activities, it is a DNA polymerase that can be used to fill in gaped DNA substrates, it is a 3' to 5' exonuclease that will degrade 3' terminal damaged DNA (i.e. and abasic site that has been nicked on the 3' end of the ribo-sugar), and it has a 5'-3' exonuclease activity that works in conjunction with the DNA polymerase activity to simultaneously polymerize a DNA strand while degrading any damaged DNA located 5' to a nick. With the addition of T4 DNA ligase, the reaction now has the ability to fill in gaps and to remove damaged DNA located on either side of a nick, followed by sealing of the strand by the ligase activity. We did find that many damaged samples improved in the STR analysis protocol after treatment with these two enzymes (data not shown). We also determined that the ratio of DNA polymerase and DNA ligase in this reaction was quite important, and there needed to be an excess of ligase. This reaction contained the reaction buffer which was optimal for these two enzymes. Since these two enzymes are the basis for our repair reaction and there are many different nucleases for evaluation, all of our subsequent work uses this reaction buffer.

We next started to evaluate improvement in our STR analysis results with addition of various nucleases to this polymerase/ligase reaction. Different glycosylases and endonucleases were evaluated in this reaction mix for their ability to assist in the reaction scheme depicted in figure 7. Each of the enzymes in figure 2 were considered during this phase of the project, and chosen based on their ability to repair the different types of damage which are summarized in figure 8.

Figure 7: This is a reminder of the general repair scheme being developed in this effort. Each of the outlined steps in this figure are being designed to occur within a single reaction, in a single incubation step, using a blend of the different enzymes that has been optimized for robustness.

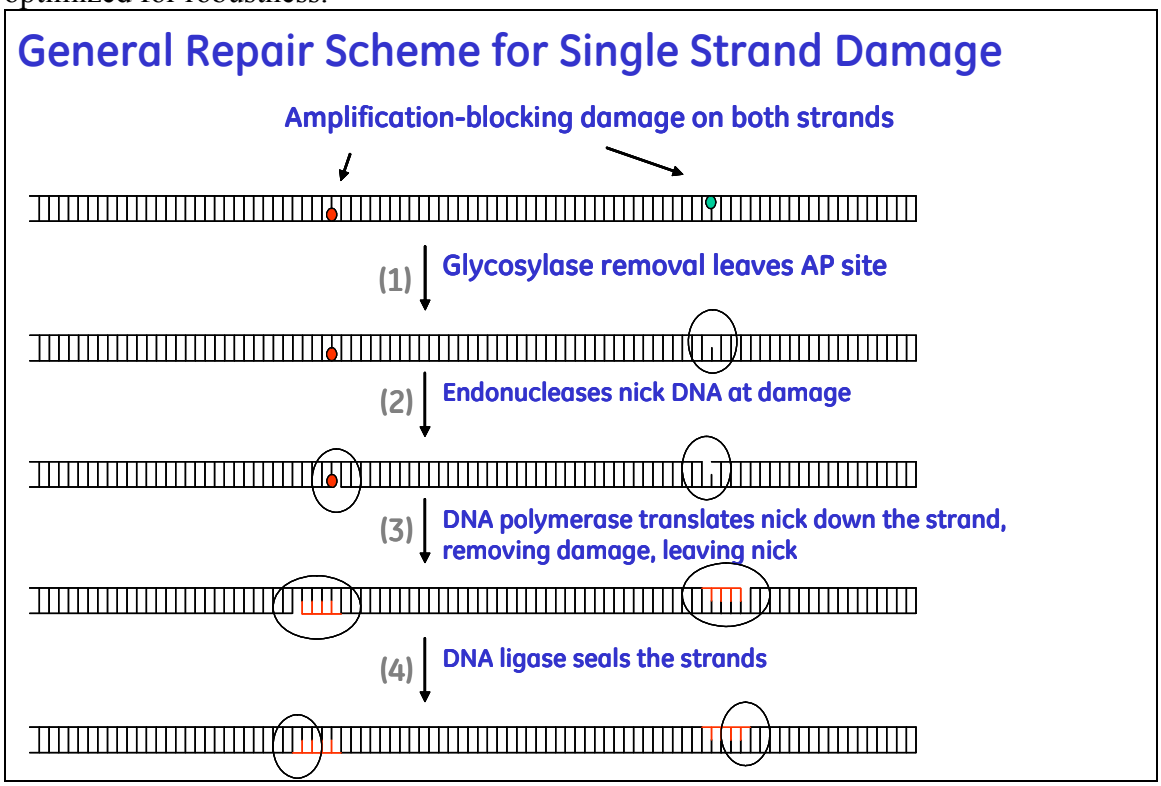


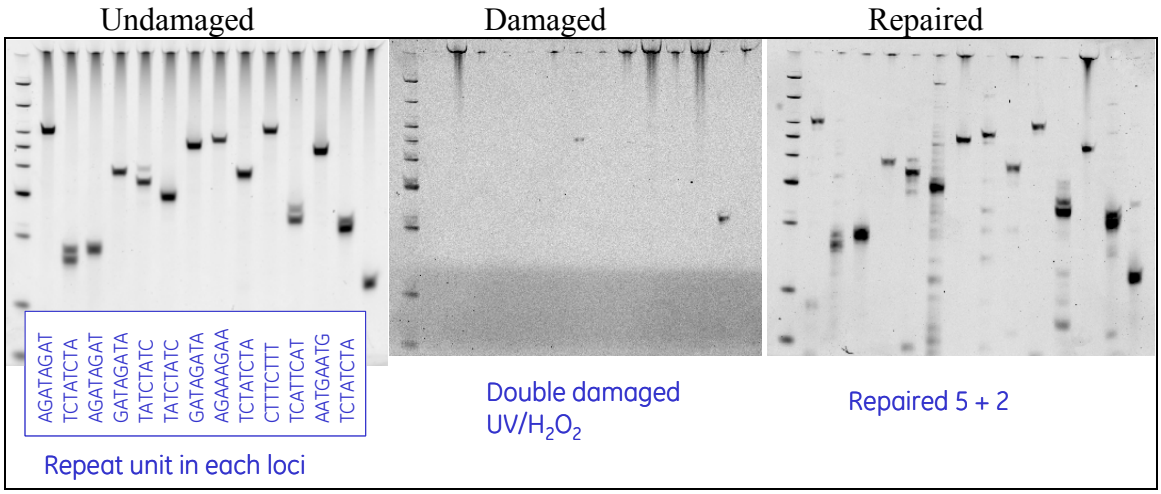
Figure 8: This is a reminder of the different types of damage that can occur within DNA samples, and possible causes for the damage. Each of these different types of damage were introduced into the DNA samples used in this study for optimization of the repair reaction.

Causes of damage

Depurination	Heat and acid
Modified base	Ionizing radiation Electrophilic agent Alkylation Fenton reaction (Fe^{2+} + H_2O_2)
Modified sugar	Same as above
Deamination	spontaneous
Linked pyrimidines	UV irradiation
Strand breaks	Enzymatic Free radical

In the following section, gel images of the profiles we observed using our STR analysis protocol are included. All of the gels in this section have been loaded in the same order and each of the PCR reactions was performed on one nanogram of starting DNA sample. Since it is difficult to describe banding patterns, background bands, band intensity, and other characteristics of these gels, we decided to include images of the gels in this report. However, there is a graphical summary of these experiments at the end of this section.

Figure 9: Typical results from undamaged DNA, damaged DNA, and successfully repaired DNA. In the first panel is the 14 loci results using undamaged DNA, in the middle panel is a partial profile resulting from profiling a double damaged DNA sample, and in the third panel of the results obtained after repair using the polymerase/ligase mixture with both E. coli endonuclease V and T4 endonuclease V (T4-PDG). In this particular figure, the sequence of the DNA repeat located in each STR was indicated. In the course of this work it was determined that certain of the loci did seem prone to damage, but no good correlation between sensitivity and sequence motif was seen. In general however, the larger loci did seem more prone to damage, most likely as a result of simply having a greater chance of being damaged because of the larger size. It should be noted that an undamaged DNA control was performed in every experiment, but in the following figures the image of this gel is not included. Furthermore, analysis of this DNA by the team at NERFI suggests that this cell line has an allelic imbalance at some loci. For the purposes of these experiments this observation has no relevance. In this figure we have included the DNA repeat sequence contained in each of these loci. It is interesting to consider the possibility that some of these repeat sequences may be particularly susceptible to damage, and that may be one reason for particular loci to dropout with even just modest damage, but careful analysis does not identify any sequence motif that could be a target for high probability damage.

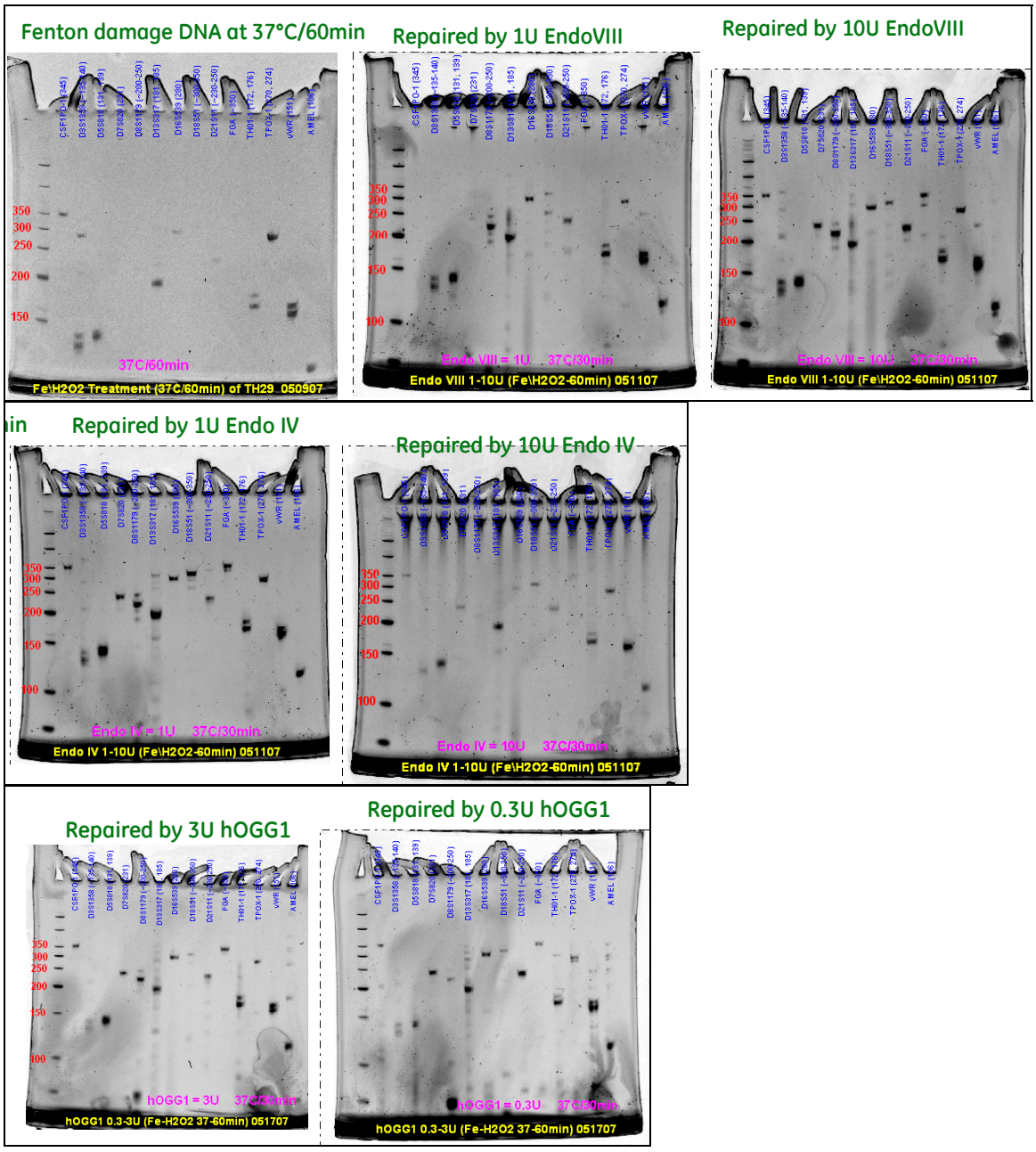


All gels shown are loaded in the same order:
Lane

1) MW marker
2) CSF1PO
3) D3S1358
4) D5S818
5) D7S820
6) D8S1179
7) D13S317
8) D16S539
9) D18S51
10) D21S11
11) FGA
12) TH01
13) TPOX
14) vWR
15) AMEL non-STR control

The size range of these individual PCR products are very similar to size range of the loci obtained using the various forensic kits sold for determining the repeat length of the 13 CODIS loci.

Figure 10: Repair of H_2O_2/iron oxidative damage. These are results obtained from analysis of the different repair reactions performed followed by PCR amplification of the 13 STR loci plus one additional control loci. A summary of these results follows later in the section. The next few figures have all been generated in an effort to optimize the various reactant components using different types of enzyme blends. It should be noted that every gel presented is loaded in exactly the same order and that all PCR reactions were done using exactly the same formulation. Additionally, for each one of the damaged samples there is a single gel showing the results obtained with un-repaired DNA followed by gel images of the different repair reactions performed. These experiments are not exhaustive, as only experiments that showed improvement in loci are shown.



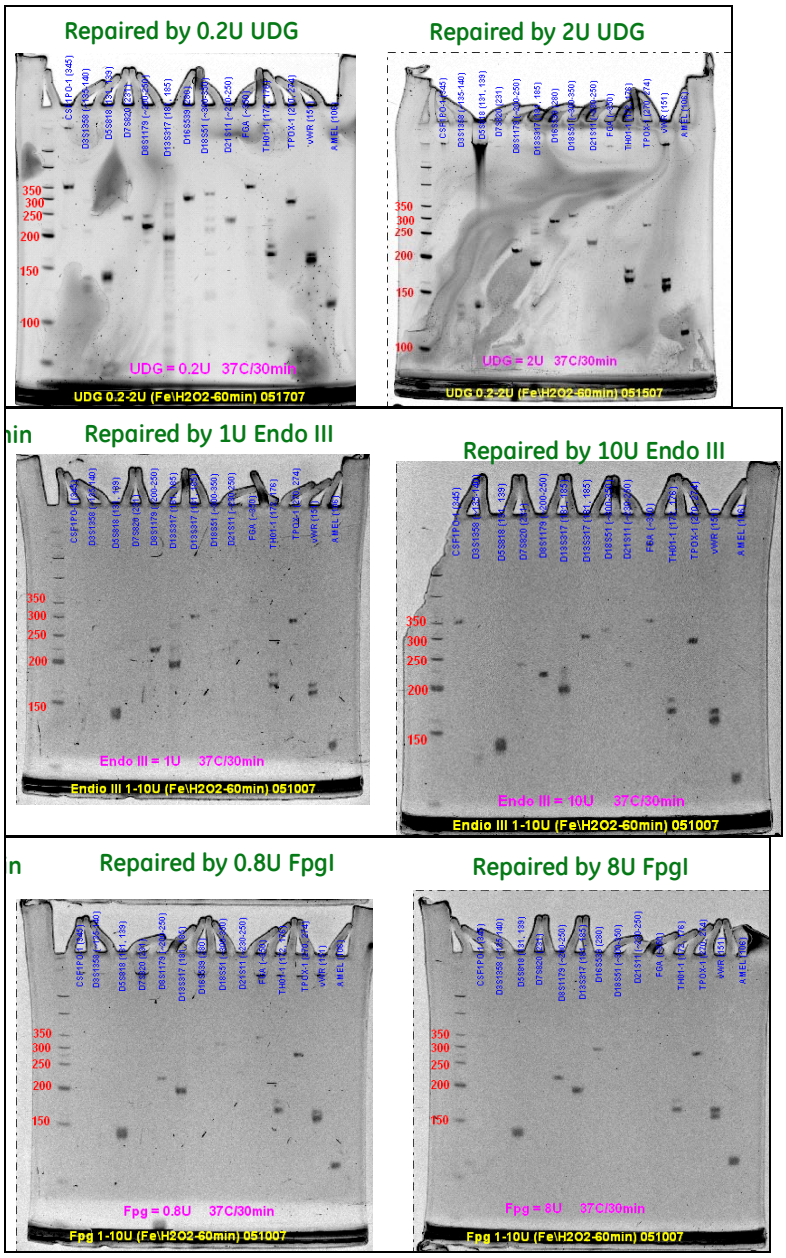
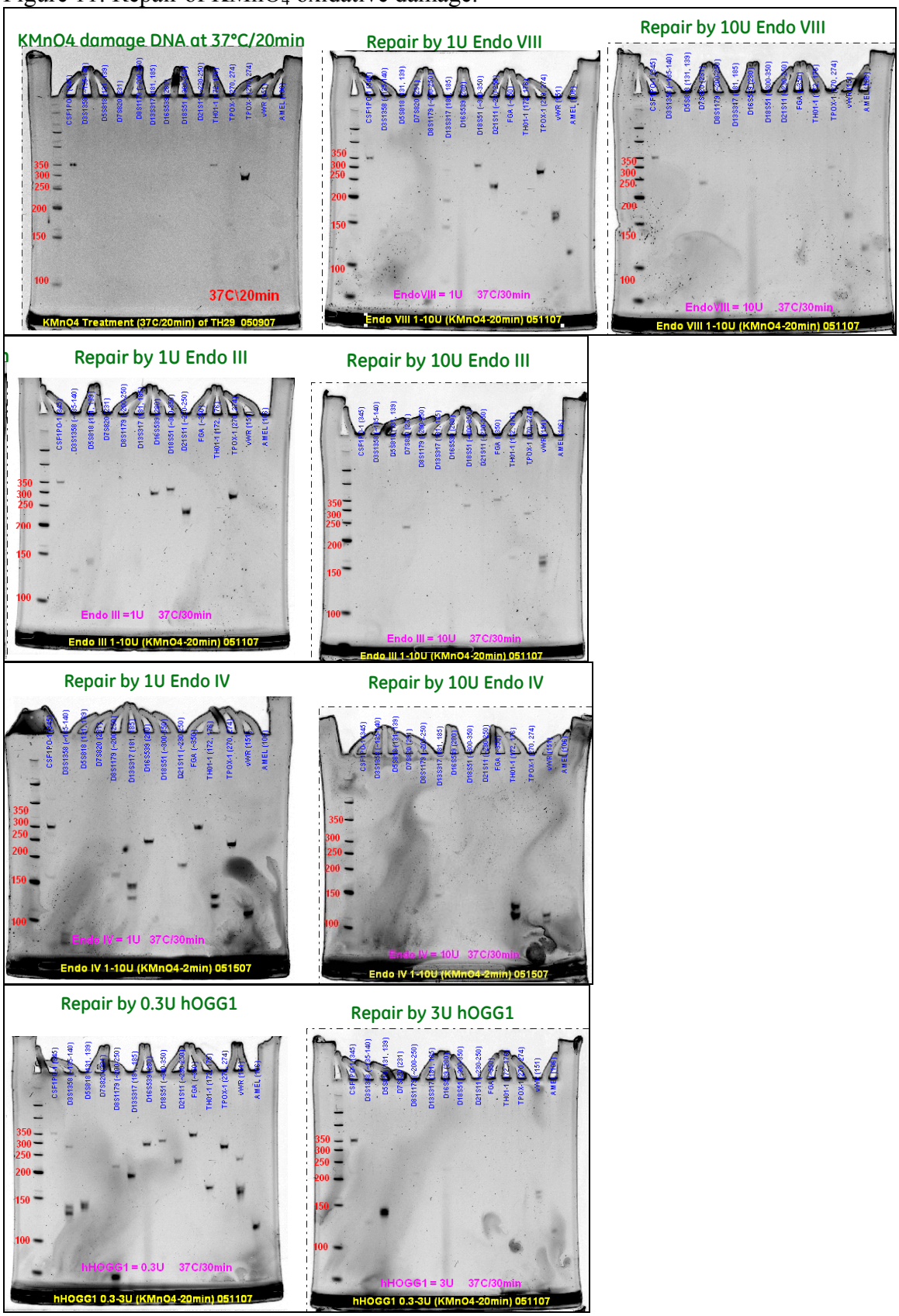


Figure 11: Repair of $KMnO_4$ oxidative damage.



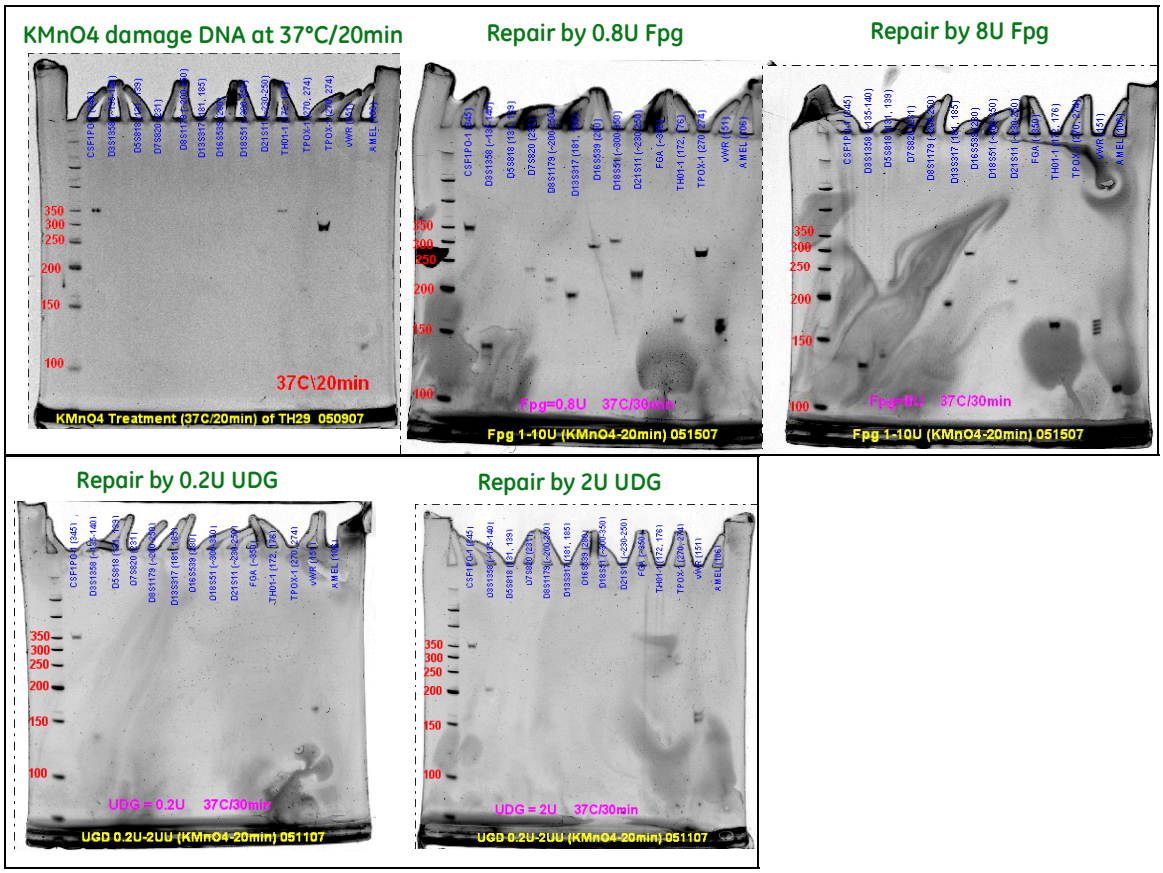


Figure 12: Repair of acid-induced damage

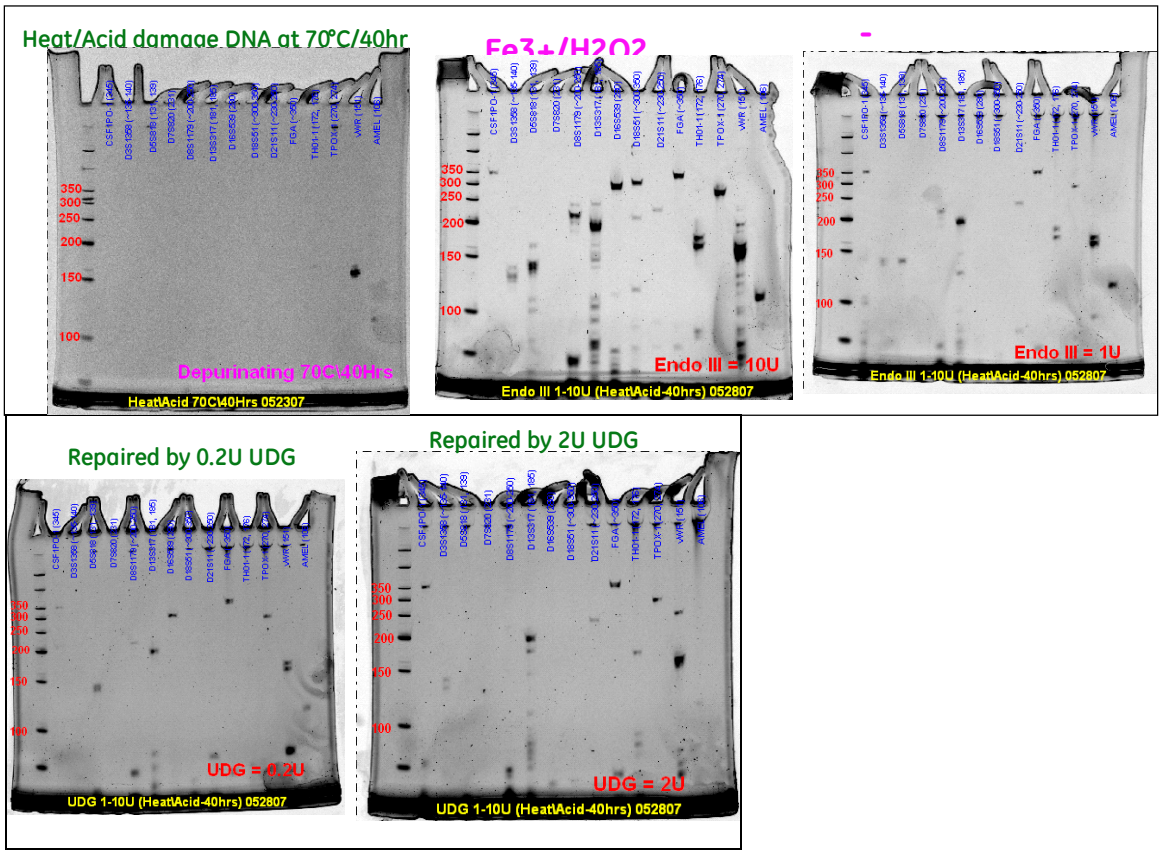


Figure 13: Repair of UV damage

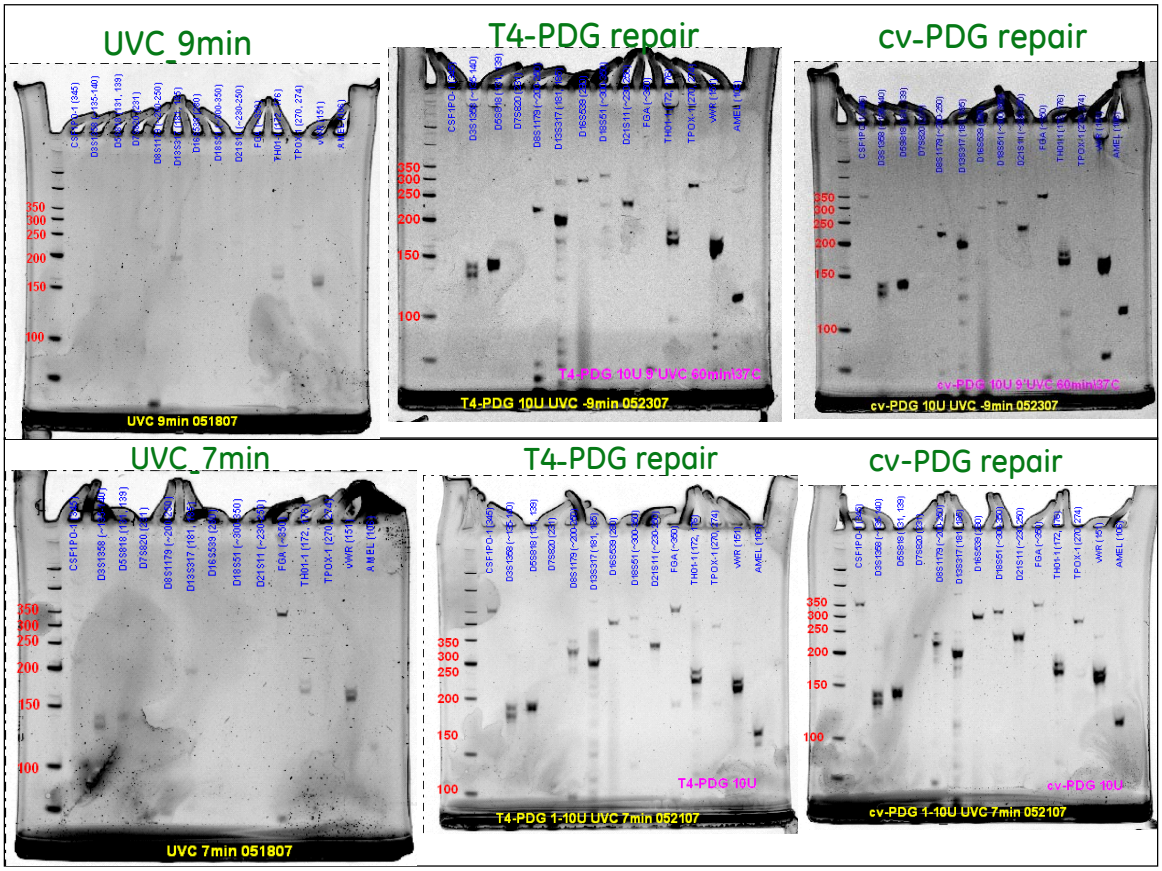


Figure 14: Repair of alkylation damage. No decrease in the number of loci was seen in the DNA that was damaged by methyl methanesulfonate. In the time course analysis the sample either gave a complete profile or totally failed.

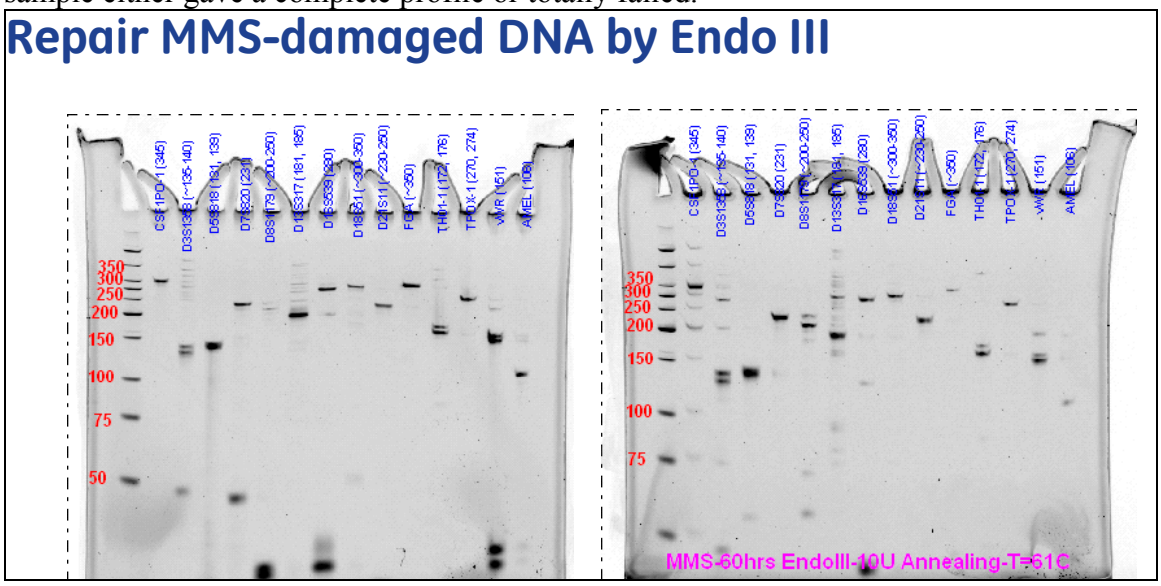
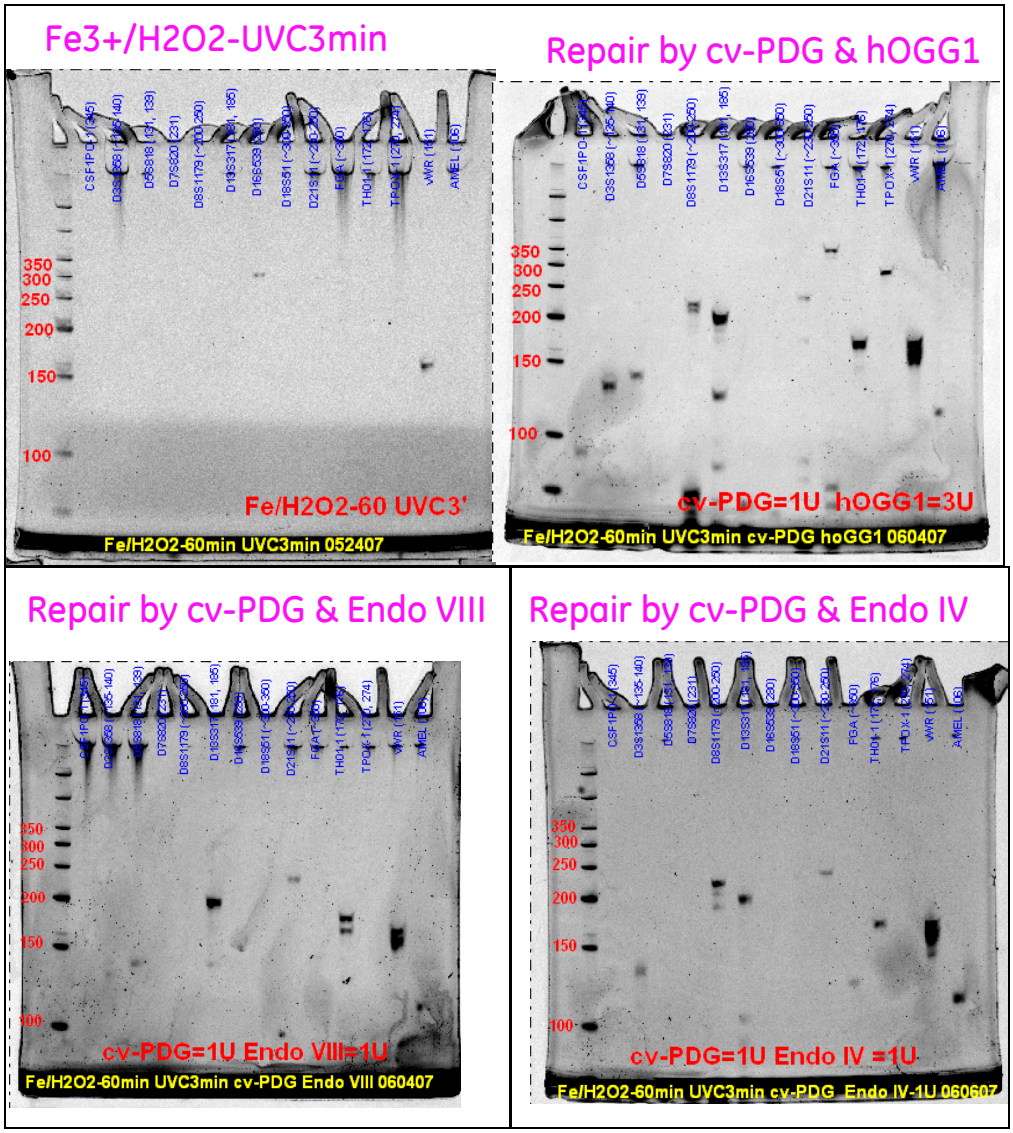
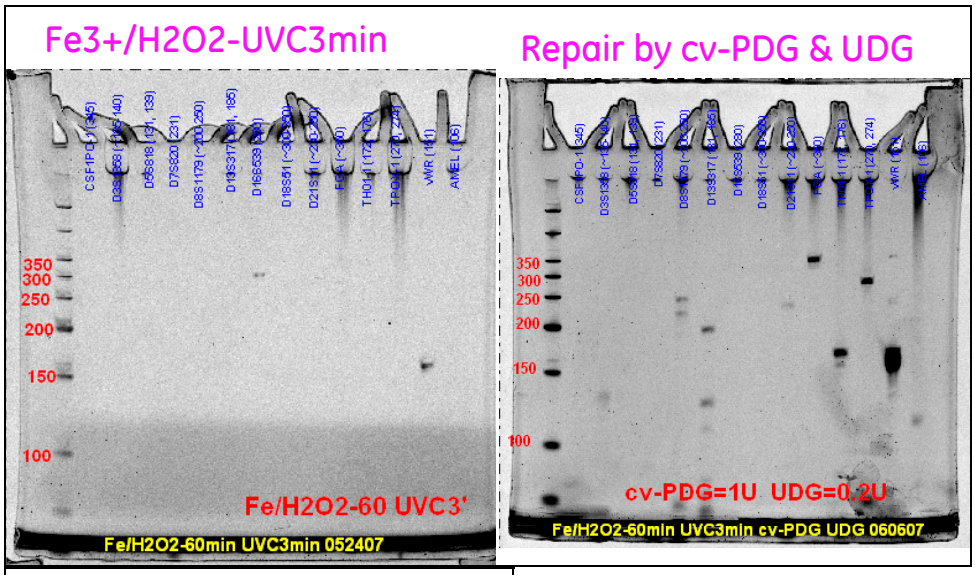
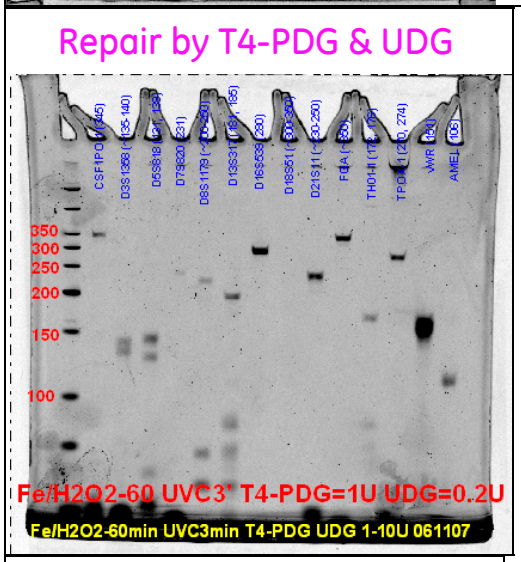
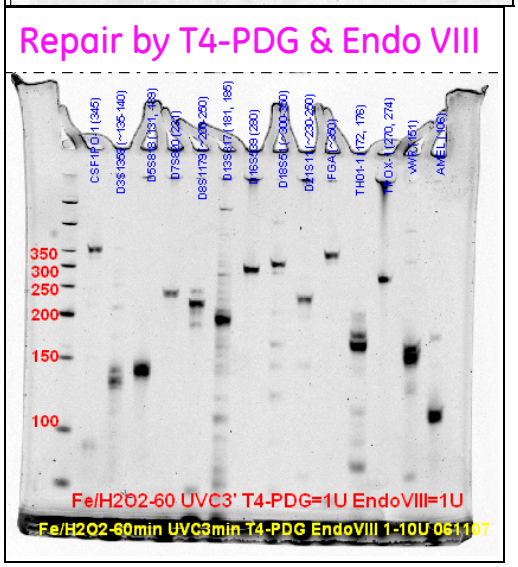


Figure 15: Repair of double damaged, UV and oxidative damage by enzyme blends

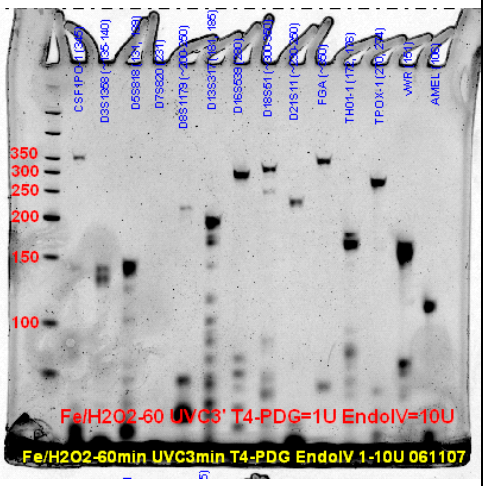




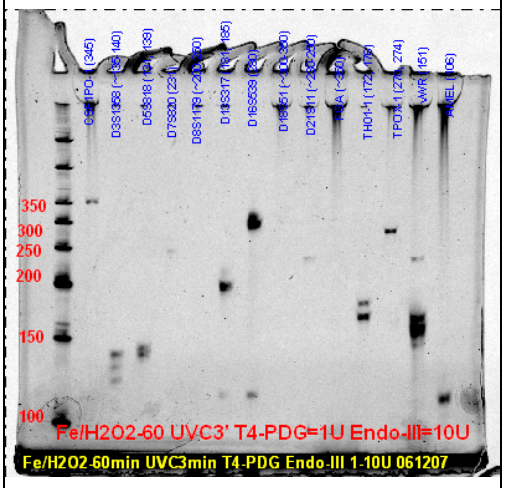




Repair by T4-PDG & Endo IV



Repair by T4-PDG & Endo III



Repair by T4-PDG & Fpg

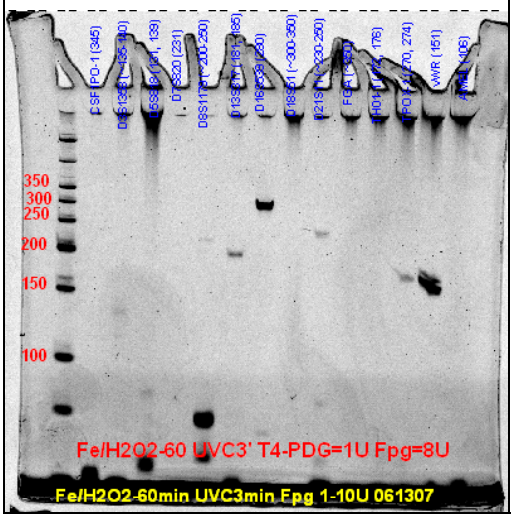


Figure 16: Repair of heat/acid damage by enzyme blends

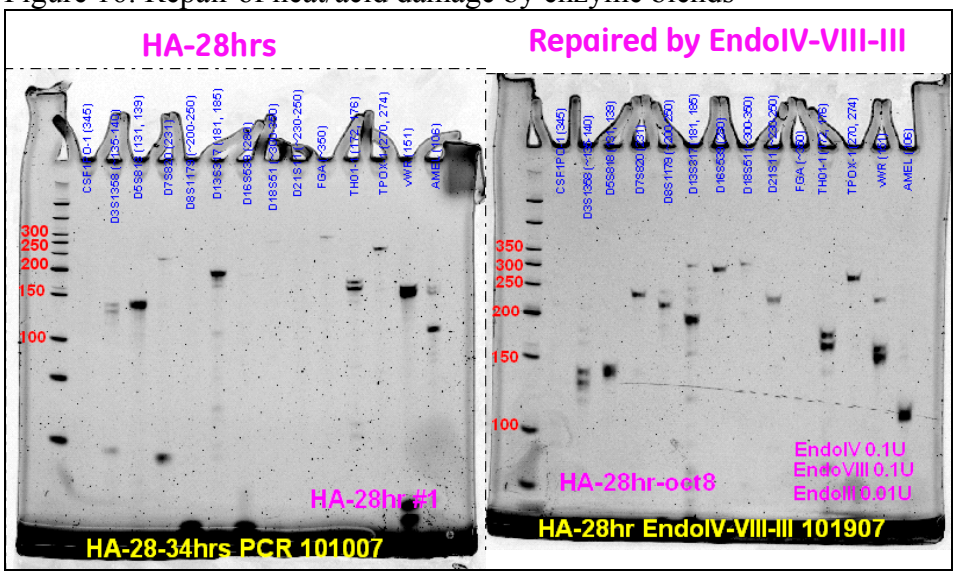


Figure 17: Repair of H_2O_2/iron oxidative damage by enzyme blends

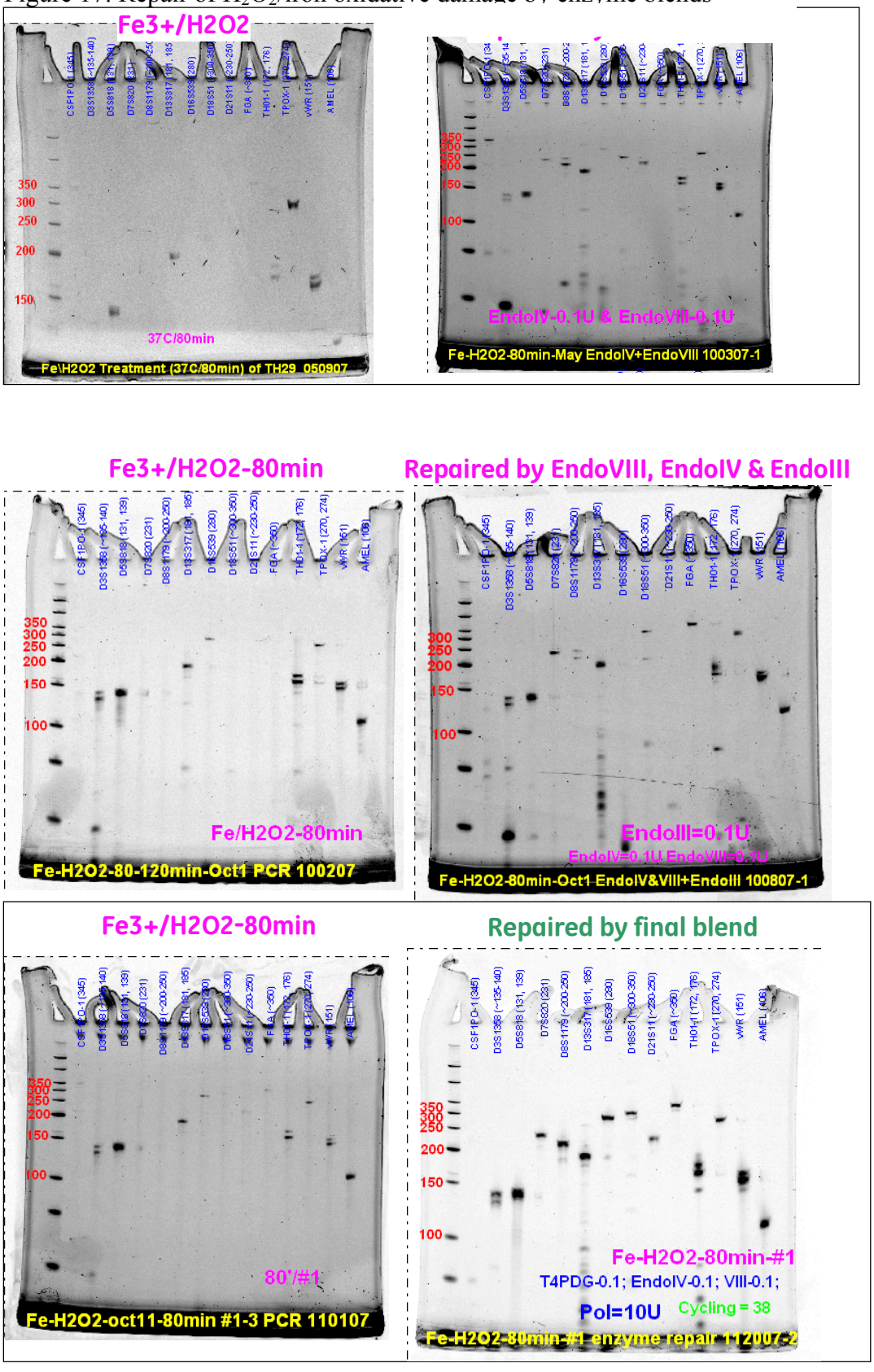
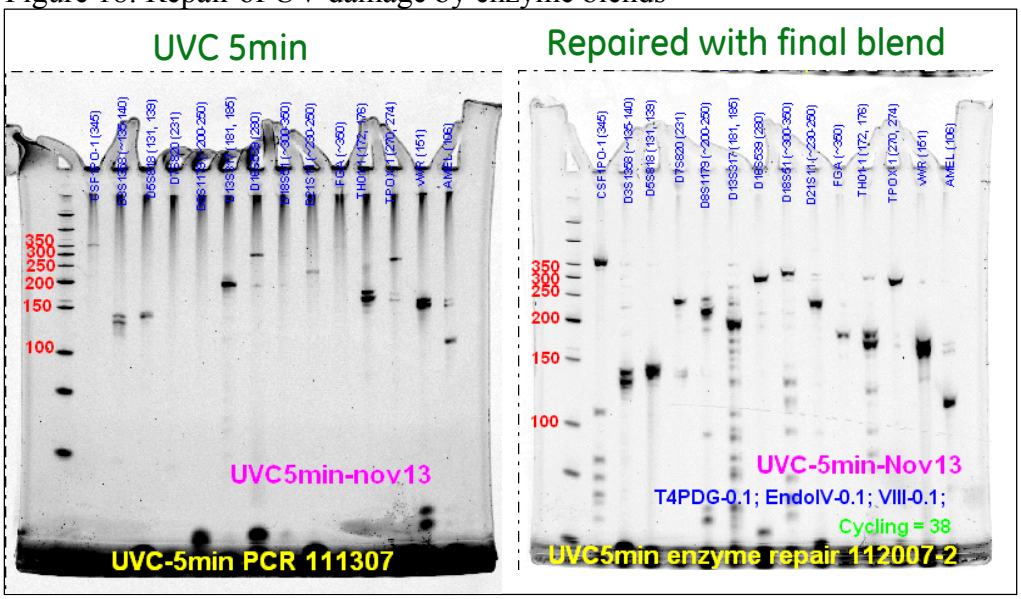


Figure 18: Repair of UV damage by enzyme blends



Graphical summary of method development work:
We have developed a single 37 degrees, 30 minute repair reaction which can be added directly to a PCR reaction which improves the number of alleles that can be obtained from DNA that is damaged by oxidation, acid or UV light.

For the STR-PCR experiments described, the number of alleles identified for the damaged (un-repaired) DNA (depicted in red in each panel, damaged as indicated) and then the number of alleles called for the repaired samples (depicted in green) were collected and graphically illustrated below. The positive control is not shown, but in all cases gave a perfect score of 14. This graphical summary is simply an interpretation of the gel images shown above (figures 9-18). Results with suboptimal enzyme levels and results with enzymes that did not improve the number of loci scored have been eliminated to allow for easier comparison. In the following figures, the reactions which contained the enzymes that were eventually chosen for the final formulation have been indicated using circles or **bold font**. Of note, when the appropriate amount of the repair enzymes is used, there is never a decrease in the number of alleles successfully amplified.

number	enzyme identity
1	endonuclease III
2	**endonuclease IV**
3	**E coli endonuclease VIII**
4	HOG/ 8-oxoguanine DNA glycosylase
5	**T4 PDG**/ T4 Endonuclease V
6	cv-PDG/ Chlorella Virus Pyrimidine Dimer Glycosylase
7	UDG Uracil DNA Glycosylase
8	**FPG/ E. coli 8-oxoguanine DNA glycosylase**

Figure 19: Repair of UV damage

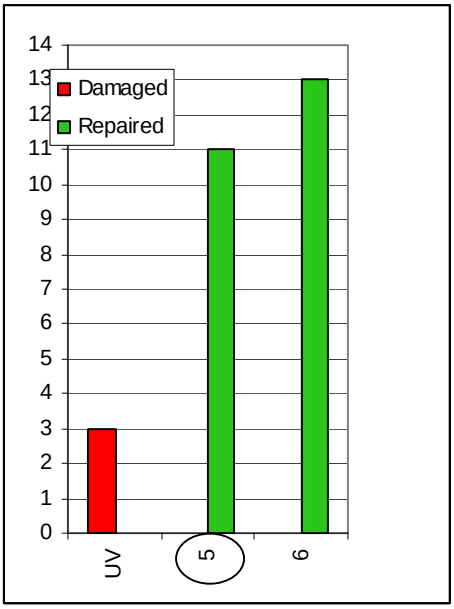


Figure 20: Repair of H_2O_2/iron oxidative damage

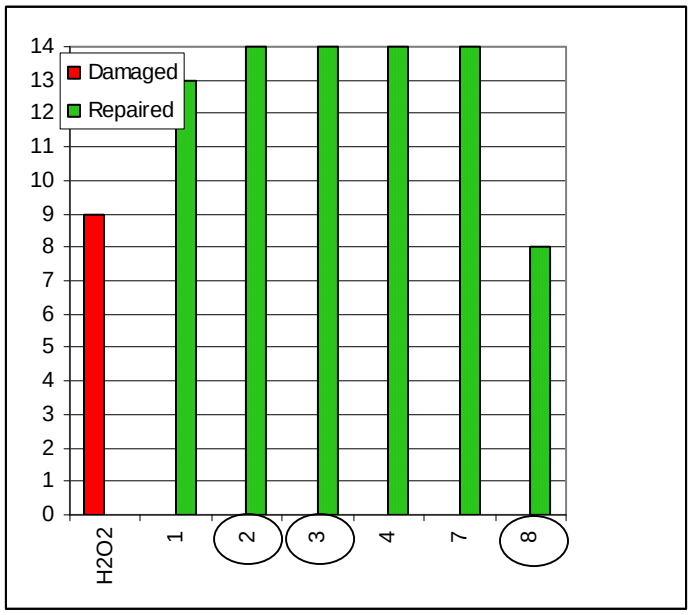


Figure 21: Repair of heat/acid damage

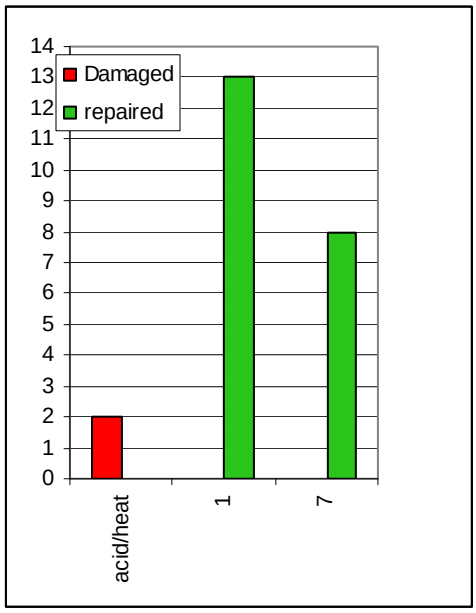


Figure 22: Repair of $KMnO_4$ oxidative damage

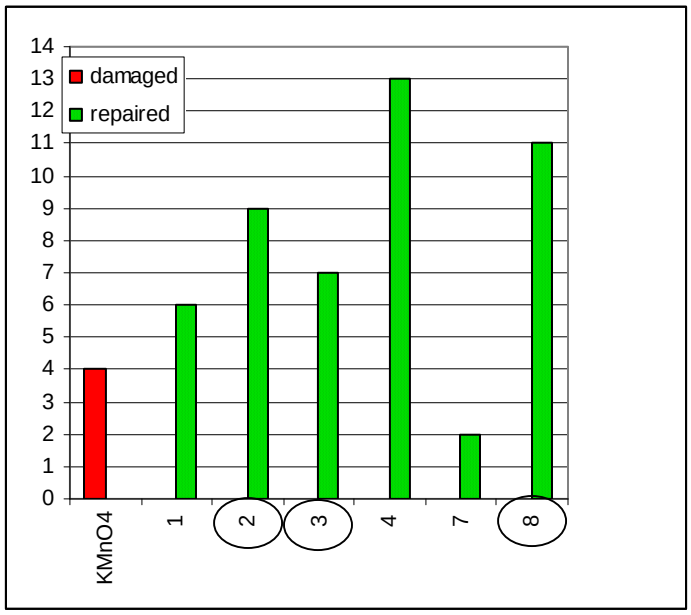


Figure 23: Repair of H_2O_2/iron and UV double damage by enzyme blends

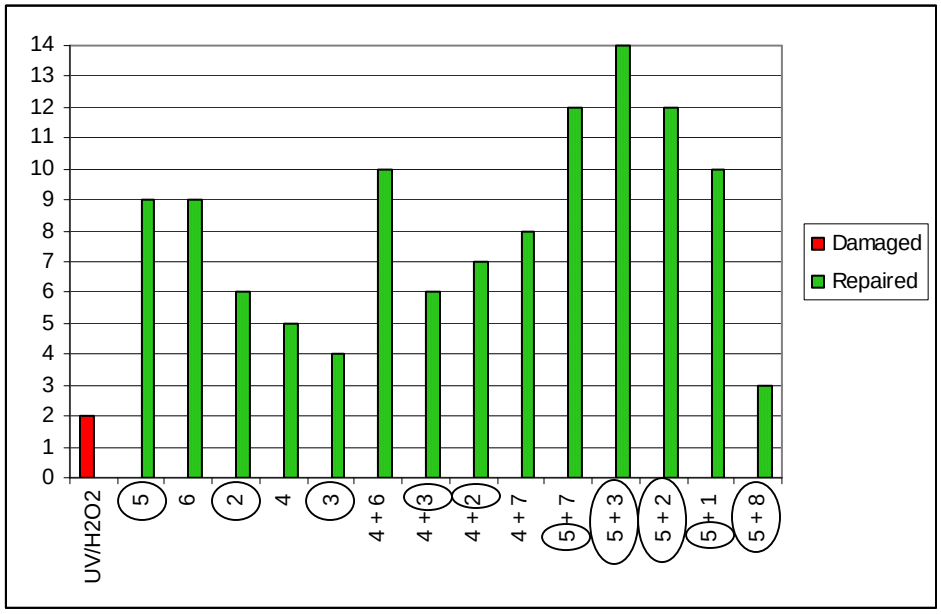


Figure 24: Repair of H_2O_2/iron oxidative damage by enzyme blends. Different samples of damaged DNA were used, so the un-repaired sample (red bar) was repeated for each sample

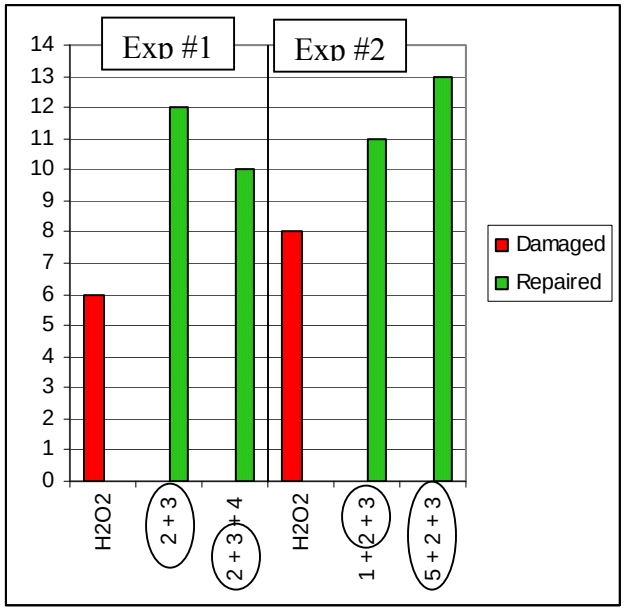


Figure 25: Repair of heat/acid damage and UV damage by enzyme blends

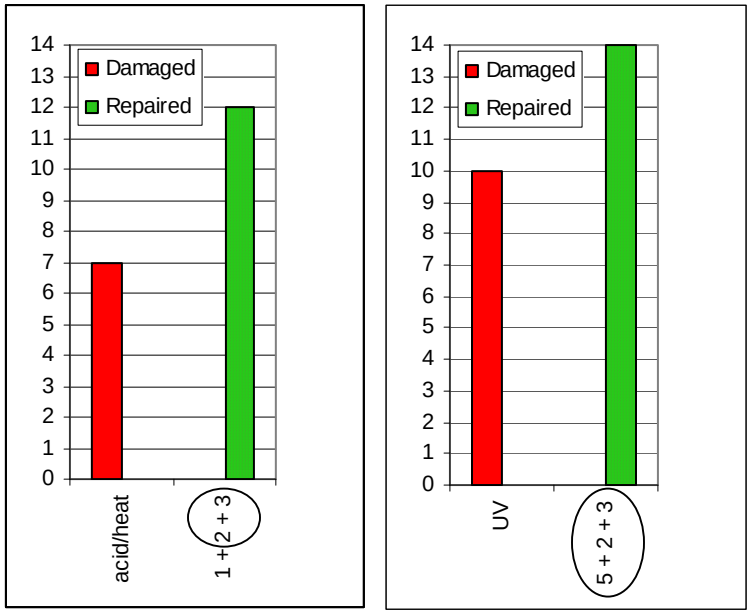


Table 3: Summary of repair of the oxidative damaged DNA - single enzyme repair

Enzymes	Repair for Fenton reaction damaged DNA	Repair for KMnO4 damaged DNA
Endo VIII	10U – recover all the loci- Excellent! 1U – recover most of the loci – Excellent!	1U – repair partial of the loci. 10U- no repair!
Endo IV	10U – recover few of the loci – not recommend 1U – recover all the loci – Excellent!	1U – repair most of the loci – Very good!
Endo III	10U – recover most of the loci – good! 1U – recover few of loci – Not recommend	1U – repair partial of the loci. 10U- no repair
Human-hOGG1	Both 0.3U and 3U repair all the loci – Excellent!	0.3U - repair almost all the loci – Excellent!
Fpg	Not much recovery – not recommend	0.8U - repair most of the loci – very good!
UDG	2U – pair partial of loci – fair 0.2U – repair all the loci – Excellent!	No repair!

Table 4: Summary of repair of oxidative and UVC damaged DNA by mix of UV & oxidative repair enzymes

	cv-PDG	T4-PDG (T4 Endonuclease V)	No second enzyme
Endo IV	Very little repair. poor	Repair all the loci. excellent	Few repaired.
Endo VIII	Very little repair. poor	Repair all the loci! excellent	Few repaired.
hOGG1	Repair some of STR loci. fair	Repair most of the STR loci. good	Almost no repair.
UDG	Very little repair. poor	Repair most of the STR loci. good	
Endo III	----------	Repair some loci. fair	
Fpg	----------	Repair few loci poor	
-----	1U cv-PDG: repair some of the STR loci.	1U T4-PDG: repair some of the STR loci.	

Summary of repair of damaged DNA with multiple enzymes

- If the type of damage has been identified, the use of a single enzyme to repair the damage is the best choice.

- The single damaged DNA can be repaired by a mixture containing multiple repair enzymes, but the amount of each enzyme needs to be carefully formulated.

- The best formulation for use: multiple enzymes to repair damaged DNA:

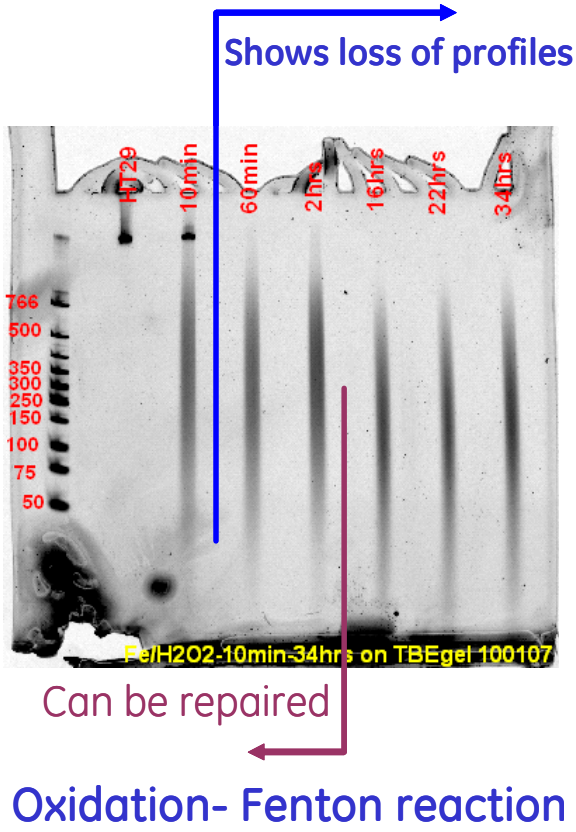
In a 30 ul reaction

Required:	10 units	E coli DNA polymerase I
	400 units	T4 DNA ligase
	0.1 units	T4-PDG
	0.1 units	Endonuclease VIII
	0.1 units	Endonuclease IV
optional:	0.008 units	formamidopyrimidine [fapy]-DNA glycosylase (Fpg)

If a reaction smaller than 30 microliters is desired the number of units of each of these enzymes needs to be scaled so that the concentration of enzyme stays the same as is in this 30 microliter reaction. We have determined that 10 microliters of this mixture can be added to a 25 microliter Identifiler reaction with no detrimental effects. So if the DNA concentration is unknown and it is a trace sample, a 10 microliter repair reaction can be used and added in its entirety to the PCR reaction.

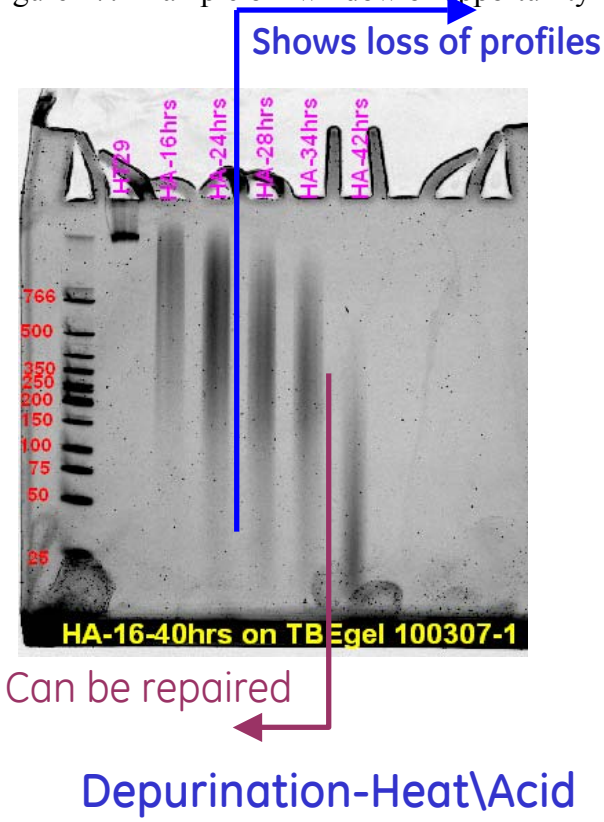
The mixture of T4 DNA ligase and E coli DNA polymerase I (plus dNTP's and rATP) to proofread/nick-translate/ligate the damaged DNA is essential. In some cases we find that this reaction alone can provide for significant improvement in DNA quality.

Figure 26: Example of "window of opportunity" for DNA repair



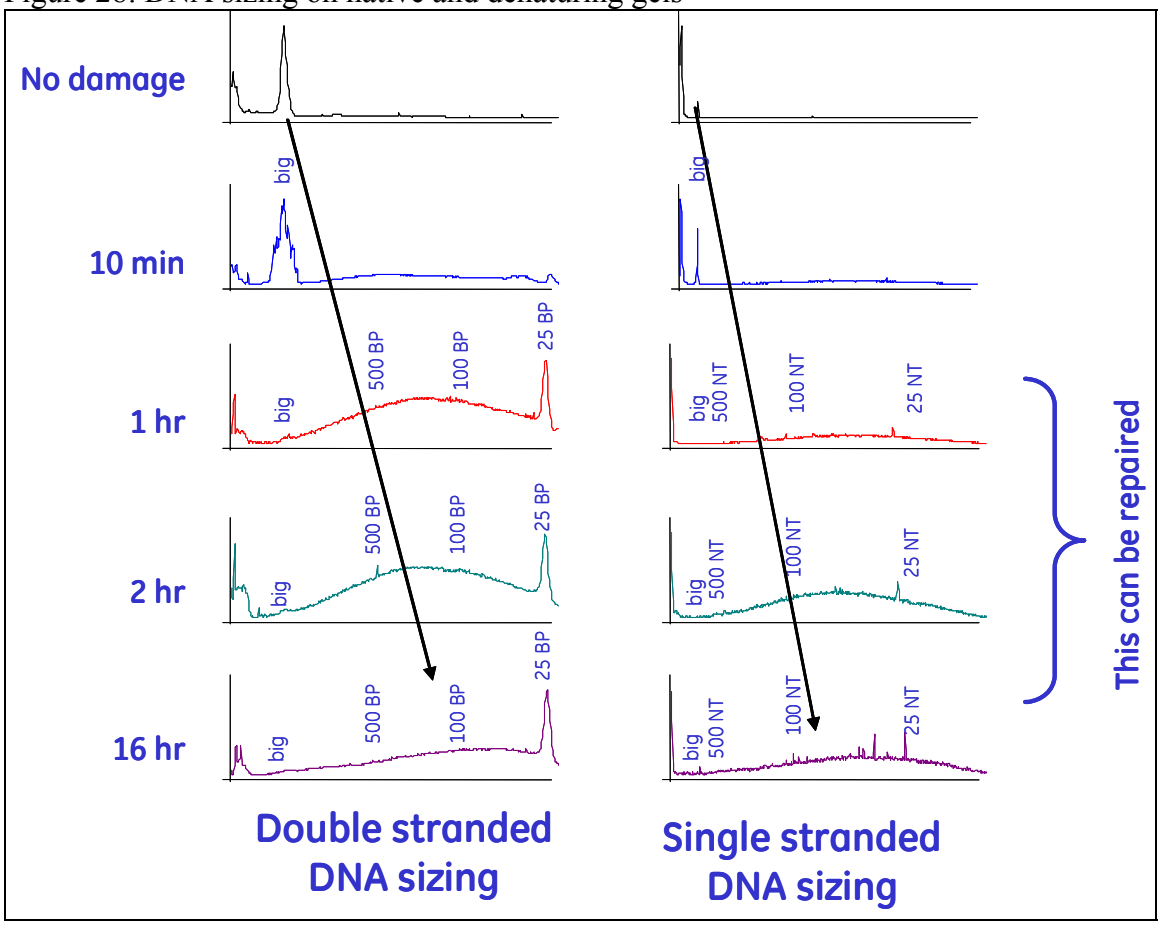
In this experiment, the DNA sample was treated for greater lengths of time with oxidizing conditions (the mixture of peroxide and metal is a commonly used reaction to damage samples by oxidation). As the duration of damage is increased there is a corresponding decrease in the molecular weight indicative of DNA degradation occurring as the DNA is damaged. When these samples were subsequently tested for the ability to be repaired, a subset of the intermediately damaged samples gave improved STR profiles after repair (data not shown).

Figure 27: Example of "window of opportunity" for DNA repair



In this experiment, the DNA sample was treated for greater lengths of time with heated acidic conditions (which typically results in depurination of the DNA). As the duration of damage is increased there is a corresponding decrease in the molecular weight indicative of DNA degradation occurring as the DNA is damaged. When these samples were subsequently tested for the ability to be repaired, a subset of the intermediately damaged samples gave improved STR profiles after repair (data not shown).

Figure 28: DNA sizing on native and denaturing gels



GE Results summary

The GE-GRC team first developed a nick translation and ligation repair reaction using E coli DNA polymerase I and T4 DNA ligase. This mixture was demonstrated to efficiently repair DNA that had been damaged by nicks, a very common issue. This reaction is the basis for all subsequent repairs that will use endonuclease to nick DNA at the site of damage. This basic polymerase/ligase mixture was demonstrated to provide moderate repair under some circumstances without the addition of endonuclease.

The team then used a model system comprised of human DNA that been damaged under controlled laboratory conditions using single damaging conditions. These DNA samples were used in STR analysis to demonstrate loss of STR profiles. The damage included UV damage, oxidative damage, alkylation damage and acid damage. Enzyme cocktails were then optimized and compared on each of these control DNA samples for their ability to repair the DNA and improve STR scores.

Once each type of damage was repairable individually, combinations were of damage were used to test the various mixtures of repair enzymes and optimize conditions to allow for repair of the most wide range of DNA damage types. The goal was to develop a

reaction mixture that could repair any type of DNA damage (other than double strand breaks).

The team has concluded that, as predicted in the proposal, there is definitely a "window of opportunity" for DNA repair. As a DNA sample is increasingly damaged, there seems to be concurrent degradation that occurs. That is, while some of the DNA becomes modified in way that can be repaired, there are a number of double stranded DNA breaks that occur at the same time. These double strand breaks can not be repaired and prevent STR typing. We find, however, that if an un-repaired sample can give a partial profile, our repair method does frequently increase the loci that are scored correctly. As the damage to samples was increased, there comes a point when there is complete failure to improve STR results, as predicted with samples that are simply destroyed.

Interestingly, during the course of the funded project, a kit called "PreCR" was launched by New England Biolabs that is designed to repair damaged DNA samples. This kit was also tested, and while it performed well with respect to repair and increased loci, it resulted in occasional drop-in loci, certainly something to be wary of (data not shown). In side by side comparisons, the final formulation developed in this project seemed to outperform the PreCR kit, certainly with respect to drop-in alleles which we have never seen with our formulation, but a larger scale comparison was not attempted due to funding and time limitation.

One side note, since forensic samples are quantified using a PCR-based test (Quantifiler), and this test essentially determines the concentration of a specific size fragment of human DNA. The size of the Quantifiler locus is smaller than the 13 STR loci (62 bp loci for quantifiler vs 106-330 bp for Identifiler). We find that any STR loci that are larger are typically the first to fail as DNA is damaged or degraded. Our conclusion is that partial profiles could potentially be eliminated by reformulating the Quantifiler kit to test for/quantify a locus which is larger than any of the STR loci. While this would eliminate many damaged samples from being tested in the first place, any sample that did pass the new long-Quantifiler test might very possibly give a complete STR profile.

The goal of NERFI's participation in this study is to validate the utility of the enzyme repair cocktail developed at GE using simulated forensic samples.

Work at NERFI:
NERFI results:
Cell Line DNA

NERFI Laboratory—Using 1 ng of template DNA for the STR amplification followed by a standard 5 s sample injection for CE, no profiles were developed for the UVC-damaged (5 and 7 min) DNA, the cv-PDG-repaired 5 and 7 min UVC-damaged DNA, the oxidation-damaged DNA, or the oxidation-damaged DNA repaired with a single repair enzyme, UDG, and the polymerase-ligase mixture (Table 5). Nevertheless, the positive control and ladder profiles were good. Therefore, CE was repeated on the same plate, increasing the injection to 20 s to determine whether the addition of more DNA in the CE would yield a profile. Thus, with the 20 s injection and 1 ng amplification, the number of genotyped loci picked up per sample ranged from 1 to 9. Amplifying the DNA at 10 ng with a 20 s injection yielded the best results—the number of genotyped loci per sample ranged from 8 to 13. Re-extracting the repaired DNA and amplifying it at 1 and 2 ng with a 20 s CE sample injection did not appear to improve the results overall (data not shown).

Table 5

Damaged and repaired HT29 cell line DNA amplified with Identifiler STR Amplification kit.

control	1 ng	10 ng
undamaged	**HT-29**	**HT-29 HI**
D8S1179	10, 16	9,10,11,13,15,16
D21S11	29, 30	29,30
D7S820	10	10
CSF1PO	11, 12	11, 12
D3S1358	15, 17	11, 15
TH01	6, 9	5.3,6,8.3,9
D13S317	11, 12	11, 12
D16S539	11, 12	11, 12
D2S1338	19, 23	19, 23
D19S433	10,14,16.2	11, 12
vWA	17, 19	17, 19
TPOX	8, 9	8, 9
D18S51	13	13
AMEL	X	X
D5S818	11, 12	6,11,12
FGA	20, 22	20,21,22

	Oxidative Damage (Fe3+/ H_2O_2)				**UVC Damage (5 min)**				**UVC Damage (7 min)**			
	Damaged		**UDG Repair**		**Damaged**		**cvPDG Repair**		**Damaged**		**cvPDG Repair**	
Locus	**20 s 1 ng**	**20 s 10 ng**	**20 s 1 ng**	**20 s 10 ng**	**20 s 1 ng**	**20 s 10 ng**	**20 s 1 ng**	**20 s 10 ng**	**20 s 1 ng**	**20 s 10 ng**	**20 s 1 ng**	**20 s 10 ng**
D8S1179	10	10	10	10	10	10	10	10		10		10
D21S11		29		29, 30	29	29, 30	29	29, 30		29, 30		29, 30
D7S820				10								
CSF1PO				11, 12		11, 12		11				
D3S1358	15,17	15, 17		15		15, 17	15, 17	15, 17		15,17	17	15, 17
TH01	6	6, 9	6	6, 9		6, 9	6	6, 9		6		6, 9
D13S317	11			11		11, 12		11				
D16S539		11		11, 12				11, 12				11
D2S1338		19		19, 23								
D19S433	14	14	14	13, 14	14	14	14	10,13.2,14,15,17.2	14	14	14	14
vWA	17, 19	17, 19		17, 19		17, 19	17, 19	17, 19		17		19
TPOX	9	8, 9		8, 9		9		8				
D18S51				13				13				
AMEL	X	X		X		X	X	X, Y		X		X
D5S818	11, 12	11, 12	11	11, 12		11, 12	11	11		11, 12		12
FGA				20, 22		20		20				

Summary of results for damaged and repaired HT-29 cell line DNA amplified at 1 and 10 ng with a 20 s CE sample injection. Alleles highlighted in yellow most likely are artifacts.

The results are encouraging, however, in that we were able to develop partial to full profiles on DNA subjected to damage and enzyme repair at GE using our multiplex STR amplification and CE.

Bloodstains

Weather Chamber

Dried blood samples were damaged by incubation in a UV weathering chamber as described. To assess damage intensity, DNA was extracted from only one of the different bloodstains after the first weather chamber experiment. Following STR amplification using an estimated 1 ng template and CE sample injection at 5s, full DNA profiles were developed for each time point. Moreover, RFU levels for the larger loci did not appear to decrease with increased exposure in the weather chamber during the first experiment.

Loss of signal intensity, or decreased peak height, at the larger STR loci is an indicator of DNA degradation or damage because the larger loci are the first to fail in PCR amplification [xiv,xv, xvi]. Chung, et. al. describe a "decay curve…in which peak height is inversely proportional to the amplicon length." For example, in a damaged or degraded sample one would expect to see higher peaks at DS81179 (with alleles sizes in base pairs in the 100's) than at CSF1PO (with alleles sizes in bp in the 300's).

Because of the lack of damage in this first sample, the remaining bloodstains from all 5 subjects were returned to the weather chamber for an additional 24 h exposure. DNA was then extracted, and STR amplification was performed with using an estimated 1 ng of template for each subject and CE was performed with a 20 s injection. Despite the additional 24 h exposure, complete profiles were obtained for all 5 bloodstains (subjects 6707A, 62107A, 62107B, 62107C, and 62107D). It should be noted that the 20s injection for the 24 h bloodstains was likely too long as all of the samples, including the reagent blank, the amplification negative control, and the positive control, exhibited pull-up from the LIZ sizing standard. Pull-up is a result of spectral overlap and can be caused by sample overloading.

To summarize, subjecting the dried bloodstains to simulated sunlight in a weather chamber did not result in DNA profile loss for any of the time-points. In retrospect, this may have been anticipated with these dried samples since it has been previously demonstrated that it took approximately 102 h of constant exposure to the most damaging kind of UV radiation (UVC) to obtain full profile loss in dried bloodstains [xvii]. In the weather chamber, which simulates natural sunlight, our dried bloodstains were subjected to visible light as well as UVA and UVB. Ballantyne suggests that the state of dehydration as well as the "cellular milieu" provides more protection to dried bloodstains that might be afforded hydrated blood or other types of biological stains.

Window Light

We then chose to pursue a course of damage similar to what might occur naturally at a crime scene, i.e., allowing the dried bloodstains to sit in a window for several weeks. Indoor conditions were chosen to minimize opportunities for microbial degradation. After 4 weeks of window-light exposure, dried bloodstains from Subjects 6707A,

62107B, and 62107D were subjected to STR amplification at 1 ng (estimated), followed by 5 and 20 s CE. The 5 s injection resulted in relatively low RFUs overall. Full profiles were obtained for Subjects 6707A and 62107B. As for Subject 62107D, one allele at each of the loci D8S1179 and FGA dropped below threshold to 46 and 35 RFU, respectively. Increasing the sample injection to 20 s restored the full profile for Subject 62107D. The 20 s sample injection increased the RFU levels overall for all three subjects. The increased sample injection time also introduced a few artifacts including elevated baseline and split peaks at the Amelogenin locus (Subjects 62107B and 62107D). Split peaks result from incomplete 3′ adenylation of the amplicons during PCR and appear as two peaks one base pair apart on the electropherogram.

After 12 weeks of window-light exposure, full DNA profiles were obtained for all 5 subjects with a 1 ng (estimated) STR amplification and 5 s sample injection. Based on a qualitative assessment of peak heights, there appeared to be no evidence of damage for this time point as well. It should be noted, that clear window glass allows up to 72% of UV radiation in the 300–400 nm wavelength range to pass through[xviii]. Presumably, therefore, most of the UV radiation reaching our indoor samples is comprised of UVA.

Saliva

Weather Chamber

Saliva can be an important source of biological evidence. In a study comparing DNA recovery from saliva deposited on various beverage containers, it was shown that higher DNA yields were obtained from soda cans than glass bottles[xix]. Therefore aluminum beverage cans (soda cans) were chosen for both of the weather chamber experiments as well as the window-light experiment.

Following the first weather chamber experiment ("WC-1"), DNA from saliva samples SS1–SS10 and saliva control samples S1, S2, and S3 were amplified using 10 µL of sample (unquantified). CE of the amplified saliva DNA was performed at 20 s. With a 20s injection, the samples and assay controls appeared to be overloaded. There were three allele calls just above threshold in the reagent blank from the DNA extraction of the WC-1 samples. The amplification negative control exhibited pull-up from the LIZ size standard. There were also numerous artifacts on the electropherograms from exposed samples SS1-SS10 and non-exposed control samples S1, S2, and S3 including pull-up, elevated baseline, and stutter peaks (off-ladder alleles).

Disregarding the artifacts, full profiles were developed for samples SS1 (1 min), SS3-SS5 (20, 20, and 50 min, respectively), and SS9 and SS10 (2 min and 165 min, respectively). Allele drop-out was present in samples SS2 (2 min) and SS6-SS8 (50, 105, and 105 min, respectively)(Table 6). It may seem counter-intuitive that a full profile was developed after 165 min of exposure (SS10) and not after 2 min (SS2). It should be noted, however, that these samples were not quantified and it is possible that the partial profiles could be a function of input DNA quantity and as well as damage.

Since it was possible that the samples were overloaded as a result of the injection time, the CE plate was subsequently re-run with a 5 sample injection in an attempt to reduce or remove the artifacts and thus provide a clearer picture of our results.

Interestingly, SS6 and SS7, which developed only partial profiles after the 20 s injection, had full profiles after the 5 s injection. With the exception of one allele, the profile for SS8 was also restored after the 5 s injection (Table 6).

	SS1 1 min	SS2 2 min	SS3 20 min	SS4 20 min	SS5 50 min	SS6 50 min	SS7 105 min	SS8 105 min	SS9 2 min	SS10 165 min
20 s Inj.	Full	Partial (8)	Full	Full	Full	Partial (2)	Partial (5)	Partial (5)	Full	Full
5 s Inj.	Full	Partial (6)	Full	Full	Full	Full	Full	Partial (1)	Full	Full

Table 6. Comparison of DNA profile development (full or partial) with 20 and 5 s sample injections following STR amplification of saliva samples exposed in a weather chamber for 1 to 165 min. The number in parentheses indicates the number of loci in which allele drop-out was observed.

Nevertheless, based on a qualitative assessment of decreasing peak height at the larger loci, there appeared to be some evidence of damage or degradation. However, after WC-1, it was discovered that some of the cans had not been stored in the dark prior to exposure. The box in which some of the cans had been stored was placed on a high shelf with the lid inadvertently left open. It has been shown that indoor lighting, including the fluorescent lighting typically present in office buildings, emits UVR[xx]. Indeed, some fluorescent lighting was shown to emit wavelengths shorter than those present in the natural sunlight that reaches the earth's surface (i.e., UVC). Although we do not know for sure, it is possible that the previous exposure to fluorescent lighting also had an impact on the profiles we observed.

Therefore, when the bloodstains were returned to the weather chamber for a 24 h exposure, two beverage cans used on the preceding evening (one each from Subjects A and X) were also subjected to 24 h exposure. Following STR amplification (10 µL of sample, unquantified), CE on the saliva samples was performed with a 20 s injection only. A full profile was developed for Subject A. For Subject X a partial profile was developed with allele drop-out at 6 loci. Again, artifacts including pull-up from the LIZ size standard (possibly related to the injection time) were present in the electropherograms.

Window Light

Eight cans each from Subjects A (A1-A8) and Z (Z1-Z8) were exposed to window light for 4 weeks. Following STR amplification (10 µL of unquantified sample) and CE using a 5 second sample injection, full profiles were developed for samples A1-A6, and A8. The fact that there were absolutely no alleles for sample A7 is likely to be due to an assay error, rather than total damage or degradation. Two of the Subject Z samples (X3 and X5) exhibited partial profile loss (10 loci and 3 loci, respectively). Most of the samples exhibited artifacts such as pull-up and elevated baseline (off-ladder alleles). For some of the samples, there were decreasing peak heights at the larger loci suggesting the possible presence of damage or degradation. However, since we do not know the input quantity of DNA, we cannot say for certain the extent to which damage or quantity affected profile loss.

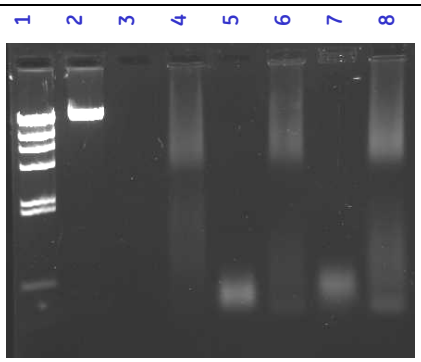
In summary, only modest success was achieved in the attempts to develop DNA damaging storage conditions of "typical" whole-cell forensic samples. The previous work done by the GE team to develop conditions that would damage DNA samples, after purification, are much more robust and reproducible.

NERFI results
Validation of GE-GRC final cocktail repair reaction-

Confirming damage and repair-

Control DNA from HT-29 cell line was damaged under controlled condition as described by either UV light, heat/acid or oxidation using the Fenton reaction as described in the NERFI methods section. Aliquots of the DNA were removed and repaired using the final formulation enzyme cocktail.

Figure 29: Repair of damaged DNA samples increases the molecular weight of the DNA



1. Lambda-HindIII Digest MW marker
2. 25 ng undamaged DNA
3. 25 ng UV damaged DNA
4. 18 ng UV damaged and repaired DNA
5. 25 ng heat/acid damaged DNA
6. 18 ng heat/acid damaged and repaired DNA
7. 25 ng H2O2 damaged DNA
8. 18 ng H2O2 damaged and repaired DNA

The single 37 degrees, 30 minute repair reaction increases the size of DNA that is damaged by oxidation, acid or UV as measure on denaturing gel electrophoresis. This is one indication of the success of repair.

Results indicate that as previously mentioned, as DNA samples are damaged there is a concurrent degradation that occurs that can limit the ability to repair the damage. In this example it is demonstrated that repair of these samples increases the molecular weight of the damaged samples presumably by repairing the nicks and gaps present on the double stranded segments by the polymerase and ligase mixture. As mentioned earlier, if the predominant size of the double stranded segments is reduced below the size of the STR loci to be analyzed, this analysis will fail. DNA samples must be damaged at an intermediate level in order to be good candidates for repair.

After 20 weeks of window light exposure a partial profile was obtained from the one subject (subject 62107A) samples. An aliquot of this sample was then additionally treated as described with heat/acid or H_2O_2/iron. An aliquot of the undamaged sample was also treated as described with heat/acid or H_2O_2/iron. These samples were then repaired using the final formulation blend of repair enzymes. Results from these samples indicate that while in some cases the DNA polymerase/ligase mixture alone can improve the results of the STR analysis, the full blend repair reaction containing nucleases, polymerase and ligase performs as well or better than the polymerase/ligase alone (table 7). These results also demonstrate that repair of damaged samples in a forensic environment can indeed improve the results of the analysis, and at least partially if not completely rescue samples that have been exposed to conditions that result in DNA damage.

Table 7: Summary of Identifiler STR typing kit analysis. Results clearly indicate that this method can be used in combination with standard forensic methods to improve results obtained from DNA samples that have been damaged by any of a variety of different environmental factors.

Undamaged DNA

	No Repair control	Pol/Lig	Blend
D8S1179	15	12,15	12,15
D21S11	31.2	28,31.2	28,31.2
D7S820	8,11	8,11	8,11
CSF1PO	12	10,12	10,12
D3S1358	16	16	16
TH01	6,9.3	6,9.3	6,9.3
D13S317	12	12	12
D16S539	12	12	12
D2S1338	16	16,17	16,17
D19S433	14	14	14
vWA	14,18	14,18	14,18
TPOX	9,10	9,10	9,10
D18S51	12,15	12,15	12,15
AMEL	X,Y	X,Y	X,Y
D5S818	10,12	10,12	10,12
FGA	21	21,24	21,24

heat/acid damaged

	No Repair	Pol/Lig	Blend
D8S1179	OL	12,15	OL,12
D21S11		28,31.2	
D7S820			
CSF1PO			
D3S1358		16	16
TH01		6,9.3	6
D13S317		12	
D16S539		12	
D2S1338			
D19S433		14	14
vWA		14,18	
TPOX		9	10
D18S51			
AMEL		X,Y	X,Y
D5S818		10,12	10
FGA			

Oxidative damage

	No Repair	Pol/Lig	Blend
D8S1179	OL		OL,12,15
D21S11		31.2	28
D7S820			
CSF1PO			10
D3S1358		16	16
TH01		6,9.3	9.3
D13S317		12	12
D16S539			12
D2S1338			16
D19S433	14	14	14
vWA		14	14,18
TPOX			9,10
D18S51			
AMEL		X,Y	X,Y
D5S818		12	10,12
FGA			24

UV damage
(20 weeks window light exposure)

	No Repair	Pol/Lig	Blend
D8S1179	OL	12,15	12,15
D21S11		28,31.2	28,31.2
D7S820		8,11	8,11
CSF1PO		10,12	10,12
D3S1358		16	16
TH01		6,9.3	6,9.3
D13S317		12	12
D16S539		12	12
D2S1338		16,17	16,17
D19S433		14	14
vWA		14,18	14,18
TPOX		9,10	9,10
D18S51		12,15	12,15
AMEL		X,Y	X,Y
D5S818		10,12	10,12
FGA		21,24	21,24

UV (20 weeks) + heat/acid damage

	No Repair	Pol/Lig	Blend
D8S1179	OL,12,15	OL,12,15	12,15
D21S11	28,31.2	28,31.2	28,31.2
D7S820			
CSF1PO			
D3S1358	16	16	16
TH01	6	6	6,9.3
D13S317	12	12	12
D16S539	12	12	12
D2S1338			16,17
D19S433	14	14	14
vWA	14,18	14,18	14,18
TPOX	9,10	9,10	9,10
D18S51			12,15
AMEL	X,Y	X,Y	X,Y
D5S818	10,12	10,12	10,12
FGA	21	21	21,24

UV (20 weeks) + oxidative damage

	No Repair	Pol/Lig	Blend
D8S1179	OL	OL	OL,13*
D21S11			
D7S820			
CSF1PO			
D3S1358			
TH01			9.3
D13S317		9***	
D16S539			
D2S1338			
D19S433			14
vWA			
TPOX			
D18S51			
AMEL			X,Y
D5S818			
FGA			

Next, undamaged control DNA was quantified using the Quantifiler kit, and either directly analyzed using the Identifiler kit, or repaired and then analyzed using the Identifiler kit. This comparison was performed to determine whether or not the repair reaction has a negative effect on undamaged DNA samples. As can be seen from the traces (figures 30 and 31 and table 8), the results are quite similar for the to samples. Interestingly, when the peak height intensities for the two samples are compared, the undamaged control DNA which was repaired gave a two to three fold increase single strength of each band (figure 32). It is unclear why the repaired sample had an increase in signal strength, as this DNA sample was not predicted to have any damage, but there is the possibility that the buffer carryover from the repair reaction may have stimulated the Identifiler reaction. In any case, the repair reaction did not have a detrimental effect on the undamaged sample.

All samples analyzed with a 50RFU threshold

Figure 30

Undamaged control: 10s CE injection, 1ng Amp (Quantifiler)

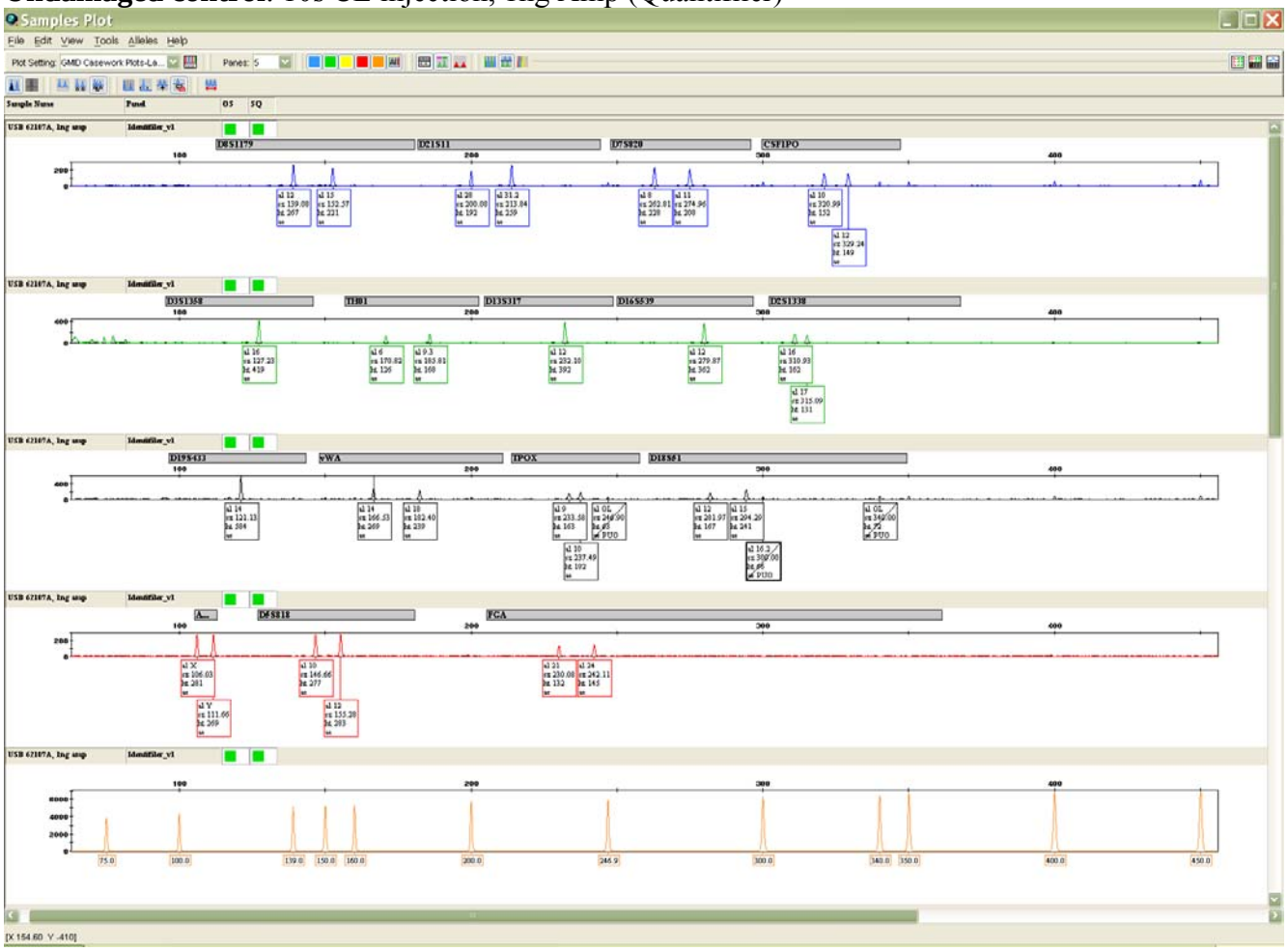


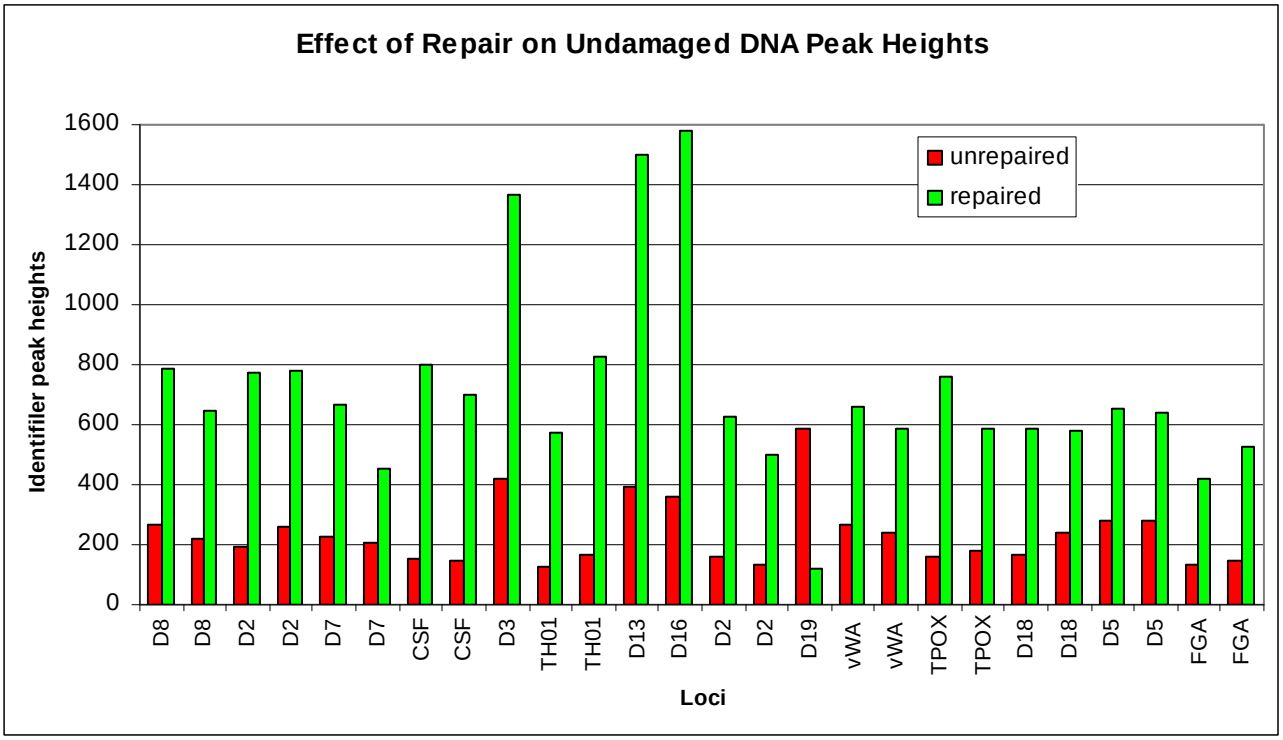
Figure 31

Undamaged control, repaired: 10s CE injection

Table 8

Identifiler Loci	Known Profile	62107A Undamaged Control	62107A Control Repaired
D8	12 , 15	12, 15	12, 15
D21	28, 31.2	28, 31.2	28, 31.2
D7	8, 11	8, 11	8, 11
CSF	10, 12	10, 12	10, 12
D3	16	16	16
TH01	6, 9.3	6, 9.3	6, 9.3
D13	12	12	12
D16	12	12	12
D2	16, 17	16, 17	16, 17
D19	14	14	14
vWA	14, 18	14, 18	14, 18
TPOX	9, 10	9, 10	9, 10
D18	12, 15	12, 15	12, 15
Amel	X, Y	X, Y	X, Y
D5	10, 12	10, 12	10, 12
FGA	21, 24	21, 24	21, 24

Figure 32



We next tested samples of pure DNA that had been damaged as part of the GE-GRC efforts, to determine if these samples behaved similarly in the single-plex PCR assay used at GE-GRC laboratory and the Identifiler PCR amplification kit used in many forensic laboratories. The 80 minute oxidized sample (see figures 2 and 17) and the 28 hr acid treated sample (figures 4 and 16) were re-quantified using the Quantifiler Human DNA Quantification Kit and a 20 µL repair reaction containing 2 ng (as determined by Quantifiler) of damaged DNA was performed. Because the quantification assay targets a 62 bp sequence, it could overestimate the quantity of intact DNA present in a specimen that can be sized using the Identifiler kit as the sizes of these amplicons can be greater than 300 bp. The repair reaction conditions were exactly as described in section 10: "final formulation" of the GE-GRC methods. After repair and enzyme inactivation by heating to 65 degrees for 20 minutes, 10 µL of repaired DNA (1 ng, as determined prior to repair) or 1 ng of non-repaired DNA was added directly to the AmpFaSTR Identifiler PCR Amplification Kit. The reaction volume was 25 µL. Results from the oxidized sample are shown in figures 33, 34 and table 8. Repair of this DNA increased the number of loci scored (RFU>50) from 8 bands to 19 bands. Results from the oxidized sample are shown in figures 35, 36 and table 9. Repair of this DNA increased the number of loci scored (RFU>50) from 3 to 16. In general the banding pattern looks normal, with no increase in baseline and no evidence of allele drop-ins. In fact, the results from a negative control repair reaction are shown in figure 37. We would advise anyone attempting to prepare this type of repair reaction to always include this negative control.

Figure 33
Oxidation damaged 10s CE injection, 1ng amp (Quantifiler)

Figure 34
Oxidation damaged and then repaired: 10s CE injection, 1 ng amp

Table 8

Identifiler Loci	Known Profile	Oxidation Damaged	Oxidation Repaired
D8	10, 16	10	10
D21	29, 30		30
D7	10		10
CSF	11, 12		
D3	15, 17		15, 17
TH01	6, 9	6	6, 9
D13	11		11
D16	11, 12		
D2	NA		
D19	NA	14	14
vWA	17, 19	17, 19	17, 19
TPOX	8, 9	9	8, 9
D18	13		13
Amel	X	X	X
D5	11, 12	11	11, 12
FGA	20, 22		20, 22

Figure 35
Heat/acid damaged: 10s CE injection, 1ng amp (Quantifiler)

Figure 36

Heat/acid damaged and then repaired: 10s CE injection ,1 ng amp

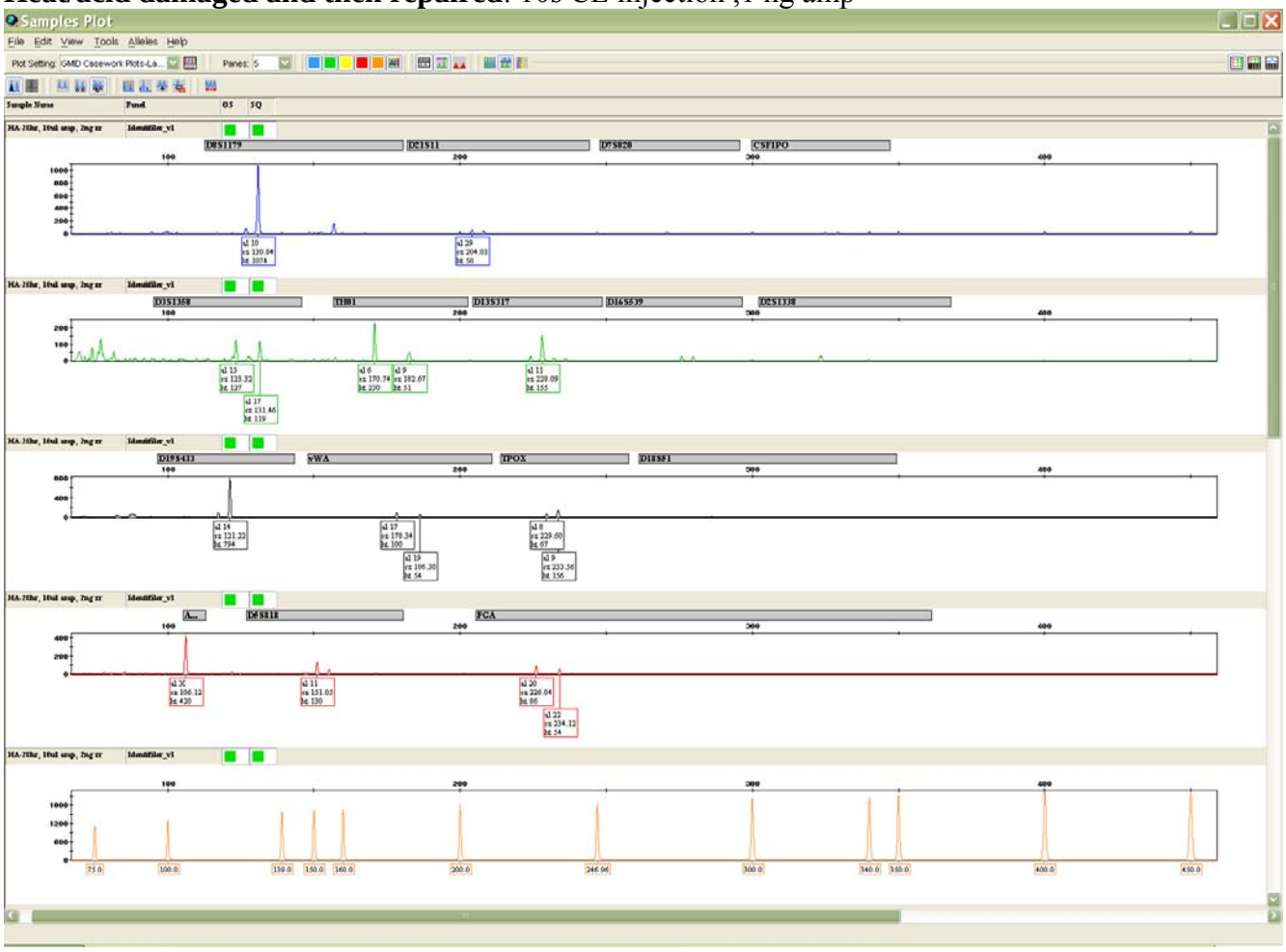
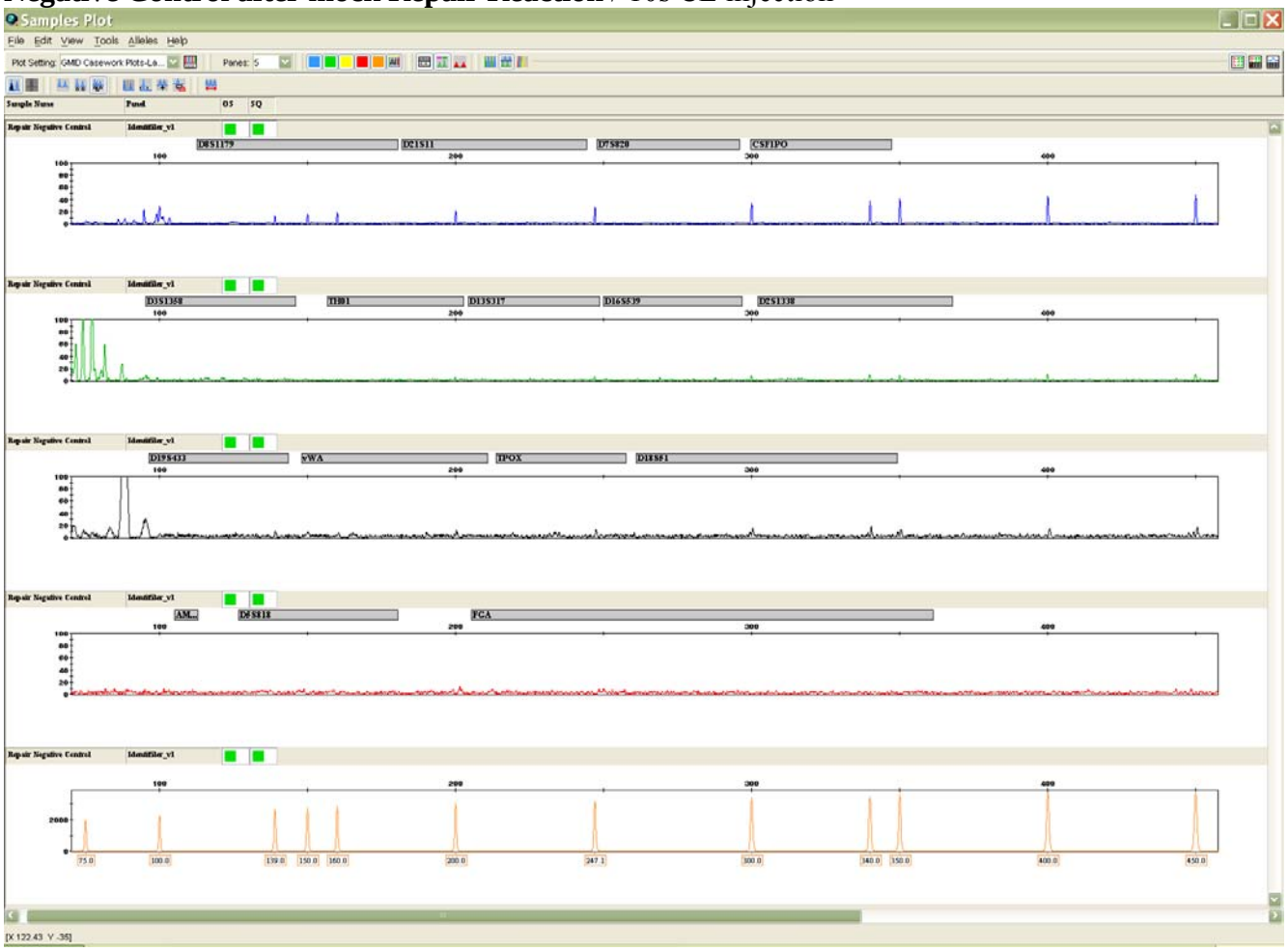


Table 9

Identifiler Loci	Known Profile	Heat/acid Damaged	Heat/Acid Repaired
D8	10, 16	10	10
D21	29, 30		29
D7	10		
CSF	11, 12		
D3	15, 17		15, 17
TH01	6, 9		6, 9
D13	11		11
D16	11, 12		
D2	NA		
D19	NA	14	14
vWA	17, 19		17, 19
TPOX	8, 9		8, 9
D18	13		
Amel	X	X	X
D5	11, 12		11
FGA	20, 22		20, 22

Figure 37
Negative Control after mock Repair Reaction / 10s CE injection



IV. Conclusions

1. Findings:

● As DNA samples are damaged by exposure to conditions that modify the chemical structure of the DNA, which can be repaired; there is concurrent degradation, which can not be repaired.

● There is likely a "window of opportunity" during exposure of DNA to sub-optimal conditions when the DNA molecular weight is large enough to still contain segments of DNA that encompass STR loci, but contain lesions that can be repaired.

● Treatment of damaged DNA samples with a mixture of DNA polymerase and ligase can partially repair samples.

● Addition of an optimized cocktail of enzymes including Fpg (formamidopyrimidine [fapy]-DNA glycosylase), E coli endonuclease IV, E coli endonuclease VIII and T4-PDG (pyrimidine dimer glycosylase) to the DNA polymerase/ligase mixture increases the ability of the mixture to repair DNA.

● Use of this repair method in the standard forensic lab workflow, as an additional step between quantification of the DNA and PCR, can increase the number of loci successfully scored.

● The repair method is compatible with the standard workflow used in forensic laboratories. The method has no adverse effect on un-damaged samples, which suggests that the implementation of this method as a standard practice in the processing of forensic samples could be beneficial.

● While the project did develop a repair reaction that can be used successfully, one potential drawback to the method involves the potential introduction of contaminating DNA, which could result in spurious allelic "drop-ins". Since none of the enzymes used in the repair method have been validated for use in a forensic setting, additional effort would be required by the suppliers of these enzymes to provide quality control assurance that each of the enzymes used was free of contaminating human DNA. Alternatively, a quality control step could be developed that would be used in the forensic laboratory that would be used each time the repair reaction is performed to confirm that in the absence of added sample the method did not introduce spurious DNA.

2. Implications for policy and practice:

DNA amplification is one of the most important and powerful molecular biology tools of our time. Improvements in methodology related to DNA amplification consistently move through the field from research to diagnostic applications. In general, DNA analysis suffers from samples that for one reason or another have been damaged to the extent that analysis becomes difficult, if not impossible. This method will enable more forgiving, yet accurate, forensic analysis, and will benefit the entire forensic field. In addition, the life

science research community will benefit in areas where DNA damage can cause analysis issues. While the study was fairly complete, and optimization of the methodology resulted in a fairly robust procedure, a commercial launch of any product that may utilize this method will require additional efforts. In our work we did not take any special precaution to avoid or eliminate contaminating DNA that may be present in the various solutions used in the kit formulation. While aseptic techniques were utilized that prevented contamination during the preparation of the test samples and reagents used, the enzymes were all purchased from outside sources, and were not specifically designed for use in identification and analysis of human DNA. As such, methods would need to be established to confirm that individual components are free of contaminants, and potential new methods of sample preparation (e.g. for the preparation of enzymes) may need to be established to insure a robust supply line. Alternatively, if forensic scientists wanted to prepare and use the described reagents and methods manually, methods would need to be developed to adequately quality control and qualify each solution prior to use on actual samples.

3. **Implications for further research:**

Some results from this work have been captured in a patent application which was initiated prior to the work done in this NIJ-funded effort. As such, there is potential intellectual property disclosed in this report. This may affect additional research and commercialization. However, unique DNA manipulation and analysis methods are frequently covered by intellectual property, and in many cases this coverage can be leveraged to help drive successful commercialization.

A summary of this work and this detailed report will be forwarded to the Life Sciences business unit of GE Healthcare. This organization is the segment of GE that provides molecular biology instrumentation and reagents, and would be responsible for commercializing any "kit" that this method might be developed into. GE Healthcare will have their research group and marketing group review the results and determine if a business case can be made for commercialization that is within the strategic direction for the company. If they decide not to commercialize a product, GE has an active out-licensing group that would contact the relevant players in this field (ABI, NEB, Promega) to determine if there are options there.

As a final option, this work will be assembled for publication in a peer reviewed journal, and in the absence of intellectual property restrictions, individual researchers could utilize these methods as needed, with the caveat that quality control and reagent qualification methods need to be developed.

Dissemination of Research Findings:

Portions of this work have been presented at the following:
Presentation- 2007 NIJ conference, Arlington Virginia
Presentation- 2007 Northeast Association of Forensic Scientists, Bolton Landing, New York
Poster- 2007 Advances in Genome Biology Technology, Marco Island, Florida

Scientists involved in this project:

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Catherine L. Bonventre
Jamie Belrose
Donald Orokos

Special thanks to Russell Gettig (New York State Police Forensic Investigation Center) for helpful consultation and insight throughout the project.

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