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Biological Evidence

Improved Detection of Male DNA in Post-Coital Samples

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ABSTRACT

Male DNA-containing samples collected from sexual assault or homicide victims can contain very low levels of cellular male DNA admixed with a large number of female epithelial cells. This often results in failure to obtain an autosomal STR profile from the male DNA donor. Y-STR analysis can been used to overcome this problem. However there are still many instances where such an approach does not work. This is particularly so when the sample is collected many days after the incident, usually as a result of delayed reporting by a rape victim or when there is a significant time interval between death and recovery of a rape/homicide victim's body or when the samples manifest some degree of degradation. Recent technological advances in the area of DNA profiling offer the opportunity to improve the number of samples that can be successfully analyzed. Therefore it may be possible to develop strategies to overcome the problems associated with low levels of male DNA in a background of female DNA. We have developed such a method using a selective amplification of loci in Y-chromosomal genomic DNA prior to Y-STR analysis. This genomic partitioning is an effective strategy to further increase the signal to noise ratio of the Y-chromosomal DNA compared with the epithelial DNA and hence allow clear and unambiguous male profiles to be obtained. Additionally, such an approach could also be used to improve the analysis of touch or contact DNA samples, which often contain small amounts of male DNA.

In this work, we have developed a 17-locus Y chromosome specific nested PCR pre-amplification multiplex and have performed initial validation studies to demonstrate its potential utility with forensic samples. The pre-amplification takes less than 2 hours to perform and can be used in conjunction with commercially available Y-STR amplification kits. The use of the nested

PCR pre-amplification prior to Y-STR analysis allows for the recovery of Y-STR profiles from as little as 5pg of male DNA (~ 1 diploid cell) from various body fluids and tissue (blood, semen, saliva and skin). No interference from female DNA was observed even with female DNA in 100,000-fold excess of male DNA. The potential efficacy of the nested PCR pre-amplification multiplex for casework was demonstrated with the ability to recover Y-STR profiles from touch/ contact DNA samples as well as extended interval post coital samples ($\geq$ 5 days after intercourse).

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EXECUTIVE SUMMARY

1. Male DNA-containing samples collected from sexual assault or homicide victims can contain very low levels of cellular male DNA admixed with a large number of female epithelial cells. This often results in failure to obtain an autosomal STR typing from the male DNA donor. Y-STR analysis can been used to overcome this problem. However there are still many instances where such an approach does not work, due to extremely low levels of male DNA. The fundamental thesis of this project was that it should be possible to develop DNA typing enhancement strategies to retrieve the male donor's Y-STR DNA profile from some samples that otherwise would be intractable to analysis.

2. In the work described herein we sought to develop novel Y-chromosome specific amplification strategies in order to "pre-amplify" only male DNA in forensic samples. QIAGEN and UCF were responsible for the development of novel Y-chromosome specific amplifications. This included primer design and initial assay development. UCF was responsible for the optimization and validation of the developed Y-chromosome specific nested PCR pre-amplification. Bond University was responsible for the development of methods for the improved analysis of degraded DNA. The QIAGEN and Bond University Sub-Contracts were active from 10/1/2007 to 9/30/2010.

3. We were unsuccessful in our attempts to develop a "whole genome" (MDA) based Y-chromosome specific amplification for low template DNA samples.

4. We **were** able to achieve selective amplification of Y chromosomal genomic DNA by using a multiplex nested PCR amplification strategy. The selective 'genomic enhancement' achieved focused on the standard Y-STR loci used in casework. The developed method turns out to be an effective strategy to increase the amount of starting male DNA present in low template samples, thus permitting Y-STR profiles to be obtained from otherwise intractable samples. We demonstrate the successful use of this approach with as little as 5 pg of input male DNA. In all experiments, we demonstrate an improvement in allele recovery as well as allelic signal intensity for samples that had been previously pre-amplified with the nested PCR multiplex. The specificity of the pre-amplification multiplex was demonstrated by the lack of interfering female amplification products. The presence of overwhelming amounts of female DNA in admixed male/female DNA samples did not impede the recovery of male DNA profiles.

5. In an attempt to increase the recovered amount of input male DNA available for Y-STR amplification, the use of DNA extract concentration and purification was evaluated. The concentration of an entire DNA extract into 10 – 12 μl permitted the amplification of all of the DNA in the original extracts. This additional concentration and purification step was utilized in the evaluation of mock casework samples including laser capture micro-dissected buccal cells, touch DNA samples and environmentally compromised samples. Probative partial profiles were obtained using as little as a single buccal epithelial cell after pre-amplification. Full profiles were recovered from contacted or touched objects such as door handles and coffee cups. Despite the larger amplicon sizes of the pre-amplification products, an improvement in allele recovery for samples exposed to 37^{o}C, 56^{o}C and storage outside (exposed to heat, light, humidity and rain) was demonstrated.

6. The use of the pre-amplification multiplex, in combination with extract purification/concentration, resulted in a significant improvement in the time frame in which male DNA profiles could be recovered from extended interval post coital samples. We demonstrate the ability to obtain full and potentially still-probative partial Y-STR profiles from samples collected up to 9 days after intercourse. This is a significant improvement in the ability to obtain probative genetic information from late reported sexual assault samples and could have an impact on the time frame in which sexual assault evidence can be collected.

7. We (i.e. Bond University) were unable to develop novel strategies to enable the restoration/repair of degraded DNA samples.

I. INTRODUCTION

A. Statement of the Problem

Y-chromosome specific genetic markers have become invaluable for the identification of the genetic profile of the male component in mixed male/female specimens for those cases in which (i) the female portion is present in overwhelming quantities relative to the male and (ii) standard autosomal STR analysis fails to yield the male donor profile. However, despite the presence of potentially probative biological evidence, a number of factors may preclude the ability to obtain a Y-STR profile from the donor. It may be that the quantity of DNA present is simply below the Y-STR detection limit or that the male DNA is partially degraded such that the number average molecular weight is less than the size of the Y-STR amplimers. Without appropriate methods to permit the recovery of profiles from these samples, value probative evidence is being lost. This project sought to develop enhanced Y-STR typing methods to permit the recovery of Y-STR profiles from previously intractable samples.

B. Literature Review

DNA typing has revolutionized forensic biology and has resulted in a dramatic increase in the ability to obtain probative information from crimes involving the transfer of biological material. The adoption of PCR-based methods in particular heralded a significant improvement in the DNA typing success of real world crime scene samples. There are, however, still many situations where the evidence contains cellular material and yet it is extremely challenging to obtain a standard autosomal STR DNA profile from that sample. One such difficult evidence type comprise post-coital vaginal samples where the rape is not reported for several days, where

a deceased victim may not be discovered for several days or where the rapist simply has a low sperm count. Other types of evidence samples that contain a male/female mixture of body fluids where autosomal STR determination of the male donor can be challenging include saliva/saliva mixtures, saliva/vaginal secretions and fingernail scrapings with cells from the perpetrator and victim, and where the male perpetrator's DNA comprises a minor fraction (< 1/20) of the admixture.

In addition to unbalanced proportions of male and female DNA in mixtures there are a number of technical reasons that add to the difficulty of obtaining an autosomal STR profile for certain types of post-coital samples. These include the lysis or loss of sperm during the differential extraction process, degradation of the sample and technological limitations of the DNA typing systems. Sperm loss after intercourse is due to vaginal lavage and drainage, menstruation, and the normal intra-cervicovaginal sperm degradative changes that occur over time. As a result of the degradative process the few remaining sperm are expected to be in a structurally fragile and possibly degraded state. Analysis of the post-coital sample in the laboratory can also result in loss of sperm, particularly during the multiple manipulations required of the differential extraction process used to separate the sperm from the non-sperm DNA fractions. The vast majority of the DNA in the non-sperm fraction comes from the vaginal or rectal epithelial cells from the victim.

A major reason for failure to obtain a male DNA profile from some post-coital samples relates to the low copy number of DNA templates present as well as the detection sensitivity of the autosomal STR systems employed. Standard protocols permit the detection of as little as 50-100 pg of DNA [1-5], which is roughly equivalent to 17-33 haploid (sperm) cells. The number of spermatozoa in samples can be less than ten cells and hence below the analytical detection limit

of the system. Moreover, DNA from sperm cells that have prematurely lysed and become part of the epithelial cell fraction may be undetectable due to the kinetics of the PCR process itself. In those instances the male:female DNA ratio would be significantly less than 1:100 and the minor male component would not be detectable because the major contributor would titrate out the critical PCR reagents required for male DNA amplification [6-9].

One solution to this problem of proportionately unequalled admixed DNA has been to substitute Y chromosome markers for the standard set of autosomal markers currently employed. The demonstrated efficacy and high sensitivity of Y-STRs for discerning the genetic profile of the male donor in mixtures of body fluids has resulted in the use of these markers in many sexual assault cases [6-12]. However there remain a significant number of cases where a male profile is still not recovered. Despite the presence in a stain of potentially probative biological evidence a number of factors may preclude the ability to obtain a Y-STR profile from the donor. It may be that the quantity of DNA present is simply below the Y-STR detection limit or that the male DNA is partially degraded such that the number average molecular weight is less than the size of the Y-STR amplimers. A viable approach to overcoming these cases would be to pre-amplify the Y chromosomal DNA prior to specific Y STR loci analysis. This DNA restoration should be an effective strategy to increase the signal to noise ratio of the Y chromosomal DNA compared with the epithelial DNA and hence allow clear and unambiguous male profiles to be obtained.

Previous attempts to improve the analysis of low template DNA (LTDNA) samples have included the use of increased cycle number (ICN) [13,14], post PCR purification [15], increased injection time [16], increased annealing time [17] or whole genome amplification (WGA) strategies [18-31]. Increase cycle number methods may not be highly efficient since additional cycles at high temperatures can lead to a decrease in the efficiency of Taq DNA polymerase [32].

The post PCR purification, increased injection and annealing time measures increase the allelic signal intensities for samples that already exhibit traces of the same alleles, albeit below the instrument's limit of detection. WGA methods employ random-sequence primers and low-stringency annealing conditions to amplify large tracts of the genome in an attempt to increase the effective number of starting templates prior to any downstream analysis. Indeed, in our own previous work we developed a modified primer extension WGA strategy (mIPEP) for use with low copy number forensic casework specimens [24]. We demonstrated the ability to obtain autosomal and Y-STR profiles from as little as 5pg of template DNA, from environmentally compromised samples, and single dermal ridge patterns using the mIPEP method [24]. However, as with other WGA methods, it was designed to amplify a large portion of the DNA sequence present within a genome and was not specific to an individual chromosome.

An alternative WGA method is based on the properties of the bacteriophage ϕ29 DNA polymerase [22]. ϕ29 DNA polymerase carries out a highly processive and continuous elongation reaction without dissociating from the template, thereby generating DNA strands of greater than 70kb in an isothermal reaction. In multi-displacement amplification (MDA)ϕ29 DNA polymerase displaces the 5' end of a newly synthesized DNA strand, thereby liberating single-strand DNA product for further priming and extension and generating high molecular weight hyper-branched DNA structures. The MDA reaction is able to amplify DNA samples by up to 100,000-fold and generates microgram quantities of DNA from picogram amounts of starting template. The use of random hexamers makes the MDA reaction essentially non-specific and allows amplification of total genomic DNA. QIAGEN has developed proprietary MDA methods for forensic samples. The REPLI-g MDA method is an isothermal genome amplification utilizing a uniquely processive DNA polymerase capable of replicating 100kb

without dissociating from the genomic DNA template. The DNA polymerase has a 3' → 5' exonuclease proofreading activity to maintain high fidelity during replication and is used in the presence of exonuclease resistant primers to achieve high yields of DNA product. In addition the REPLI-g amplification enzyme is significantly more tolerant against inhibition than Taq polymerase. Additionally, novel MDA REPLI-g technologies have been developed by QIAGEN for the specific enrichment of mitochondrial DNA (mtDNA) and the amplification of highly damaged and/or degraded DNA. Due to the reported success of this approach, it was reasonable to assume that such an approach could be utilized to develop a Y-chromosome specific amplification method.

In the current work we sought to apply the proprietary REPLI-g technology, in combination with unique design components, to the development of a Y chromosome specific DNA amplification protocol for low template samples that may (or may not) also be damaged or degraded. However, the development of a MDA based Y chromosome specific amplification proved to be very challenging and despite attempts to optimize this approach, no significant enrichment of male DNA was observed with low template samples. After the evaluation of several alternative approaches, we evaluated the use of a nested PCR based approach. Nested PCR methods utilize two sets of locus-specific primers in separate first and second round amplifications. The first round amplification primers are designed to fully encompass the locus of interest and the primer binding sites for that locus. First round amplifications, therefore, produce increased amounts of the desired starting template including necessary primer binding sites for downstream amplifications. Strom et. al reported the successful use of nested PCR amplification techniques to analyze samples consisting of charred human remains and small quantities of blood [33]. While nested PCR approaches may present challenges for use with

forensic samples due to the required larger size of the first round amplification products and potential primer compatibility problems with multiple multiplex PCR systems, we were able to successfully develop a 17-locus Y chromosome specific nested PCR pre-amplification multiplex. We have performed an initial validation of the pre-amplification multiplex to demonstrate its suitability for use with forensic samples. The pre-amplification takes less than 2 hours to perform and can be used in conjunction with commercially available Y-STR amplification kits. The use of the nested PCR pre-amplification prior to Y-STR analysis allows for the recovery of Y-STR profiles from as little as 5pg of male DNA (~ 1 diploid cell) from various body fluids and tissue (blood, semen, saliva and skin). No interference from female DNA was observed even with female DNA in 100,000-fold excess of male DNA. The suitability of use of the nested PCR pre-amplification multiplex was demonstrated with the ability to recover Y-STR profiles from touch DNA samples, laser capture micro-dissected epithelial cells, environmentally compromised samples, as well as extended interval post coital samples ($\geq$ 5 days after intercourse).

C. Statement of Hypothesis or Rationale for the Research

There are many situations in which forensic evidentiary items contain cellular material but it is extremely challenging to recover DNA profiles, such as in late reported sexual assault evidence, fingernail scrapings and touch DNA samples. It is a responsibility of the forensic community to provide suitable methodologies for the analysis of the biological evidence, even if present in trace amounts, in order to assist in the resolution of criminal investigations. Therefore, the goal of this project was to determine if chromosome specific amplification methods could be developed in order to "pre-amplify" the Y chromosomal DNA prior to specific Y-STR loci

analysis. The purpose of this was to permit the recovery of a male donor's DNA profile from otherwise intractable samples.

II. METHODS

Sample Preparation

Body fluids were collected from volunteers using procedures approved by the University of Central Florida's (UCF) Institutional Review Board. Informed written consent was obtained from each donor. Blood samples were collected by venipuncture into additive-free vacutainers and 10 - 50 µl aliquots were placed onto cotton cloth and dried at room temperature. Freshly ejaculated semen was provided in sealed plastic tubes and stored frozen until they were dried onto sterile cotton swabs (full swabs (i.e. liquid semen allowed to fully absorb the cotton swab) or 10 µl aliquots dried onto cotton swab). Buccal samples (saliva) were collected from donors using sterile swabs by swabbing the inside of the donor's mouth. Skin samples were collected by swabbing skin or an object surface with a pre-moistened (sterile Millipore water) sterile swab. All body fluid samples were dried prior to storage and stored at -20^{o}C.

For the environmentally compromised samples, blood samples were collected by venipuncture into additive-free vacutainers and 50 µl aliquots were placed onto cotton cloth. Saliva and semen samples were collected into sterile 15 ml or 50 ml conical tubes and 50 µl aliquots were placed onto cotton cloth. The bloodstains and saliva stains were subjected to various environmental conditions including storage at 37^{o}C, 56^{o}C, and room temperature and storage outside covered (exposed to heat, light, humidity) and outside uncovered (exposed to heat, light, humidity and rain). The samples were stored for various lengths of time including 1 day, 3 days, 7 days, 1 month, 3 months, 6 months and 1 year. Stains were stored at -47^{o}C until needed.

DNA Isolation

DNA was isolated from the samples using both a standard organic extraction and a differential organic extraction protocol as previously described [34], as well as the DNA Investigator kit (QIACube protocol) (QIAGEN, Germantown, MD) which was performed in accordance with the manufacturer's recommended conditions. Any modifications to the standard protocol are described in the Results section. An extraction blank (all reagents used in the extraction in the absence of any biological material) was included in each extraction performed to ensure that no contamination was encountered during the extraction process. The extraction blanks were subjected to the same analysis (see below) as the samples. Contamination in extraction blanks was not observed at any point during the course of the current work.

Purification

The MinElute PCR Purification kit (QIAGEN) was utilized for DNA extract purification and concentration, as well as post-PCR purification of the Y chromosome specific nested PCR pre-amplification samples [15]. The semi-automated QIACube (QIAGEN) protocol was used for all MinElute reactions in accordance with the manufacturer's instructions. All samples were eluted using nuclease free water (12 – 25 μl elution volumes).

Quantitation

Quantification of DNA samples (2 μl) was performed using the following real time PCR quantification kits in accordance with the manufacturer's instructions: Quantifiler® Y Male DNA Quantification kit or Quantifiler® Human DNA Quantification kit (Applied Biosystems by Life

Technologies, Foster City, CA). All quantitations were performed on an ABI 7000 or 7500 real time PCR instrument.

Polymerase Chain Reaction (PCR)

Y-chromosome STR Amplifications

Y-chromosome STR analysis was performed using the AmpFlSTR® Yfiler® PCR Amplification kit (AB by Life Technologies), the PowerPlex® Y Amplification kit (Promega, Madison, WI), or the Y-PLEX™ 12 amplification systems (Reliagene Technologies, no longer available as a commercial product). All amplifications were performed in accordance with the manufacturer's instructions using ABI 9700 thermal cyclers (AB by Life Technologies). In some instances a reduced volume Yfiler® reaction volume was used (12.5 µl from the standard 25 µl). The use of reduced volume modifications are described in the Results section, where applicable (see below). All profiles obtained throughout this study were compared to the donor reference profiles in order to verify the accuracy of the obtained profiles. All profiles reported in this work were verified and matched the donors' reference profiles. Positive and negative controls were included with each amplification (positive controls consisted of male DNA provided with the kit; negative controls consisted of sterile water). All controls were verified during analysis and only data from amplifications with proper control results were accepted.

Y chromosome specific nested PCR Pre-Amplification

This 25µl reaction mix utilized the Type-It Microsatellite kit (QIAGEN) and consisted of the following: 1X Type-It Multiplex PCR master mix, 0.5X Q-solution, and 2.5 µl of a proprietary primer mix (15 primer sets to amplify 17 Y-STR loci: DYS19, DYS385 a/b,

DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635, Y-GATA-H4). The cycling conditions for the pre-amplification were: 95°C 15 min; 15 cycles 95°C 30 sec, 60°C 90 sec, 72°C 60 sec; 68°C 10 min (final extension). Positive and negative controls were included with each amplification (positive controls consisted of a male DNA standard; negative controls consisted of sterile water). All controls were verified during analysis and only data from amplifications with proper control results were accepted.

PCR Product Detection - Capillary Electrophoresis

All amplified fragments were detected with the ABI Prism 3130 Genetic Analyzer capillary electrophoresis system (AB by Life Technologies). A 1.0 µL aliquot of the amplified product was added to 9.7 µL of Hi-Di™ formamide (AB by Life Technologies) and 0.3 µL of GeneScan™ 500 LIZ® (G5 dye set) (AB by Life Technologies), GeneScan™ 500 ROX® (F dye set) (AB by Life Technologies) or ILS-600 (Any4Dye dye set) (Promega). The electrophoretic conditions used were as follows: 16 sec injection time, 1.2 kV injection voltage, 15 kV run voltage, 60°C, 20 min run time, dye set G5 (Yfiler®), F (Y-PLEX™ 12), or Any4Dye (PowerPlex® Y). All samples were analyzed with GeneMapper® Software v4.0 (peak detection thresholds of 25 RFUs; however for some experiments this threshold was modified from 20 – 150 RFUs to compare results using various thresholds).

III. RESULTS

A. Statement of Results

Y-Chromosome Specific Multiple Displacement Amplification (MDA) Pre-Amplification Assay

The first step in the development of a Y-Chromosome Specific MDA method was to develop suitable primers that would faithfully amplify a large proportion of the euchromatic region of the Y chromosome. The sequences of all commonly used Y-STRs and the genes used in male DNA quantification (SRY gene – Quantifiler® Y (AB by life Technologies); TSPY – Plexor® HY (Promega)) were retrieved from GenBank and their precise location on the Y chromosome was determined. Additionally, sequence information was obtained for 14 other Y-STR loci that are included in the Ultra High Discrimination (UHD) multiplex specifically developed at NCFS to provide additional discriminatory power. Also compiled were the sequences of 98 Y-SNP loci since they can provide some information on the bio-geographical ancestry of the donor's male lineage. While it would be ideal to selectively amplify a large proportion of the above-mentioned regions, the chromosomal regions of most importance currently are the commonly used Y-STR loci (17 Yfiler® loci, a sub-set of which are present in PowerPlex® Y) and the genes used for male DNA quantitation.

The above-mentioned loci are spread out over 2.3×10^7 bases on the Y chromosome. The average distance between adjacent STR loci was 8.0×10^5 and the average distance between adjacent SNP loci was 2.1×10^5 bases. Two different approaches were considered for the amplification of the Y chromosome. The first approach involved amplification of the whole region covering the STR and SNP loci. With the processivity of the Phi29 polymerase ($3x10^4$) and the length of the region to be amplified ($2.3x10^7$), a minimum of 8000 primers would be

needed. This number of primers far exceeds the number of primers in a random 6-mer primer set used in MDA reactions. Therefore, it was determined early on that this approach was not feasible for the specific amplification of the Y-chromosome. The second approach to primer design would be amplify all Y-specific sequences (STRs and SNPs) with individual primer sets. The total number of sequences initially examined included 30 Y-STR loci and 98 Y-SNPs (128 total loci). The number of primers necessary, allowing for a 10-fold excess of primers, would therefore be 1280. While this was a significant number of primers, it was significantly smaller than the number of primers required using the first approach (amplification of whole region).

Given the aforementioned primer design considerations, a Y-specific MDA workflow plan was developed. The basic concept of the amplification process involved a denaturation of any male DNA present in the sample followed by the addition of the REPLI-g reagents necessary for amplification. The isothermal amplification would be carried out for 8 hours at 30^{o}C. Subsequently, the DNA yield could then be assessed and an appropriate aliquot used in downstream analyses (STR or SNP typing assays). For initial evaluation of the designed primers, 10 ng of male DNA was used. For all samples, a DNA control that had not been subjected to prior Y-MDA amplification was included for comparison.

Two different approaches to primer design for individual loci were taken. The first approach utilized a single pair of primers to frame or bind just outside of each individual locus (Figure 1). Primers, designated as “framing primers” were designed for each of the 17 Y-STR loci and are mixed together into a single primer mix and used in the Y-MDA reaction. With this type of approach both specific and non-specific priming was intended. Development of these primers took a considerable amount of time. Thirty-two Y-specific primers were designed to amplify the 17 commonly used Y-STR loci. The original primer concentration used was 2.5 μM,

although various primer concentrations were tested. Two Y-STR loci, DYS 389II and DYS437, were used to assess any enrichment occurring as a result of prior Y-MDA amplification. Amplification enrichment was assessed by a ΔCt method, which involved subtracting the Ct value of the Y-MDA amplified sample from the Ct value of a non-Y-MDA amplified sample (genomic control). If enrichment was obtained using the Y-MDA amplification, then a positive ΔCt would result (Ct value of the Y-MDA sample would be lower, indicating a higher concentration than the control sample). No specific enrichment of the Y-STR loci was observed after Y-MDA amplification (data not shown). The concentration of the primers used in the Y-MDA amplification was then increased, ranging from 2.4 μM to 5.2 μM; however, still no improvement was observed (data not shown).

The second approach to primer design involved multiple primers being designed for an individual locus, referred to as "string primers" (Figure 1). With this type of approach, only specific priming is intended. Again, development of these primers took a considerable amount of time. These primers were evaluated using the same protocol as described above, including an evaluation of a range of primer concentrations. No significant enrichment was observed using the strong primers either (data not shown).

While no significant enrichment was observed using either priming strategy in this initial testing, the developed primers were only tested using a real-time PCR detection platform. We also evaluated the primers using the same amplification conditions but using a CE-based separation and detection system. The YMDA products were amplified with Y-PLEX^{TM} 12 or a singleplex reaction [7]. The RFUs of the obtained alleles were compared in order to assess any potential enrichment with prior YMDA amplification.

Four amplification protocols were developed. The first two protocols utilized the pool of string primers designed to specifically enhance DYS385. For protocol 1, a denaturation buffer was added to each sample and the samples denatured for 5 minutes. A stop solution was then applied and a reaction mix containing reaction buffer and the primer mix (string primers). The REPLI-g polymerase was added last and then the samples were amplified at 30^{o}C for 8 hours. For protocol 2, the same primer mix was used but the samples were prepared differently. For this protocol, the sample, reaction buffer and primer mix (string primers) were added and then heated at 95^{o}C for 5 min. The REPLI-g polymerase was then added and the samples were amplified at 30^{o}C for 8 hours. The other two protocols (protocols 3 and 4) were the same as protocols 1 and 2 but they involved the use of the framing DYS primers.

Initially to test each protocol, we amplified 007 (control sample in the Yfiler® kit) in the following amounts: 5pg, 10pg, 25pg, 50pg, 100pg, 500pg, 1ng and a blank. For protocol 1, DYS385 was detected in most samples, even down to 5 pg, however often with unbalanced peak heights between the two alleles (data not shown). DYS385 was also detected in all samples without prior YMDA amplification. Therefore, YMDA did not improve the sensitivity of analysis. In addition to the DYS385 singleplex, we also tried amplifying YMDA-protocol 1 samples with Y-PLEX™ 12 to see if DYS385 would be improved in a multiplex system rather than a singleplex reaction (data not shown). The use of YMDA did not help improve signal intensity or allele recovery at DYS385 for most samples (data not shown). We repeated these samples experiments for YMDA protocols 2, 3 and 4. No improvement in allele recovery or signal strength was obtained using protocol 2 (data not shown). No genotypes were recovered for any of the samples tested using protocols 3 and 4 (data not shown).

Two possible reasons could explain the limited efficacy of MDA using Y-specific primers: (1) the polymerase is highly processive and synthesizes large DNA strands from one starting point. The consequence is that during the initial phase of amplification mis-priming events result in very long displaced DNA strands (having a length of 30000-70000 nt) that bear a high probability for another mis-priming event. If the polymerase been of lower processivity the probability of mis-priming would be lower because the displaced sequence is shorter and does not contain a high sequence complexity; (2) the polymerase is not thermostable; as a consequence, the reaction temperature is low and primer sequences must be short to bind efficiently. However, short primer sequences result in more binding sites within the genome than longer primers. If the primer length was increased, priming specificity could not be improved as long as the reaction temperature was kept constant due to the thermo-lability of the polymerase. Switching to a polymerase having a better thermo-stability without reducing the processivity could potentially increase the specificity because longer primers and higher reaction temperatures could be used. However, this would require the development of novel protocols as it would no longer be considered an MDA-based assay.

In a further attempt to employ a WGA approach, we developed another enrichment strategy for Y-specific sequences. The method coupled selective DNA purification and subsequent WGA (Figure 2). The reaction starts with double stranded DNA, which is denatured in the presence of biotinylated oligonucleotides that hybridize specifically to Y-chromosome specific sequences. Different biotinylated oligonucleotides were designed that hybridize to chromosomal regions of ~15000 bp. After hybridization of the biotinylated oligonucleotides, separation of the selected sequences is accomplished through the use of streptavidin coated magnetic beads. After washing of the beads, the selected sequences are released by heat

denaturation and a subsequent WGA used to amplify the selected sequences. Several parameters were varied in order to obtain a specific enrichment of Y-chromosome specific sequences during the purification step. These parameters included the amount of DNA used for the purification, denaturation conditions with respect to buffer, temperature and incubation time, and washing conditions. None of the modifications resulted in the ability to specifically enrich the Y-chromosome specific sequences (data not shown).

Y-Chromosome Specific Nested PCR Pre-Amplification Assay

Conceptually, the above-mentioned process provided an opportunity to selective isolate male DNA physically, allowing it to be removed from any potential male/female admixed samples. However, we reasoned that enrichment of Y Chromosomal DNA could more simply and efficiently be accomplished using a Y-chromosome specific nested PCR reaction (Figure 3). With nested PCR, first round amplification is performed with a single primer set for each target. As with any standard PCR method, it is possible during this initial amplification to obtain unwanted amplification products (primer dimers, non-specific amplification due to mis-priming) which can reduce the efficiency of amplification of intended targets. However with a nested-PCR approach, the resulting first round amplification product is subjected to a secondary amplification with a second set of primers (nested primers, binding sites contained within the first round amplification products). If incorrect amplification products were obtained in the first round amplification, they would not be amplified a second time using the specific "nested" primers in second round amplifications. This improved specificity therefore can increase sensitivity as the targets of interest are amplified more efficiently (i.e. enhancement of the desired targets, Y-STRs in this case). This improvement in specificity and sensitivity therefore

may not be observed with amplifications that, for example, simply increase the number of amplification cycles of a single reaction using single primer sets. Additionally, increased cycle number reactions can result in depletion of critical PCR reagents thereby reducing the efficiency of the amplification in higher cycler numbers. This can result in little, if any, improvement in sensitivity.

Our first efforts were focused on designing highly specific first round amplification primers in the regions flanking the 17 commonly used Y-STR loci. Initially, five Y-STR loci were selected based on their inclusion in all of the commercially available Y-STR multiplex kits. These loci included DYS391, DYS393, DYS389, DYS390, and DYS19. The design of suitable first round amplification primers proved to be quite challenging due to the high homology of these specific sequences to the X-chromosome. Primer sets for DYS393 and DYS391 were designed and we performed initial testing on these two loci as a proof of concept. Initial specificity screening experiments were conducted on these primer sets using male (1 ng) and female (100 ng) DNA to determine whether the primers would specifically amplify male DNA. A weak amplification product in the female DNA sample was observed for the DYS391 primer set. However, the amplification of the male DNA sample was much stronger. No female amplification product was observed for the DYS393 primer set (data not shown). These initial results were encouraging and led us to develop and optimize a pre-amplification protocol using these two primer sets.

First round "pre-amplification" reactions were developed using the Type-It Microsatellite kit (QIAGEN). The Type-It Microsatellite kit utilizes HotStar Taq Plus DNA polymerase and a patented buffer system, which enable multiplex PCR testing to be performed without the need for further optimization of the various reaction components. A proprietary additive, Q-solution,

is also available in the kit and is designed to facilitate amplification of GC-rich sequences. The initial evaluation of the DYS393 and DYS391 pre-amplification primers included testing of a range of male input DNA (5 pg, 50 pg, 500 pg, 1ng) in order to ensure that the pre-amplification protocol was working properly.

The results of the initial evaluation of the Qiagen pre-amplification primers can be seen in Table 1. For these studies we used the Y-PLEX 12 kit from Reliagen due to its ready availability in the lab. Twenty pre-amplification cycles with either of two different primer concentrations (0.1 μM and 0.01 μM) were used. The use of the Q-solution was also evaluated. Alleles were recovered after the pre-amplification reaction with as little as 5 pg of input male DNA (Table 1). A significant improvement in allelic signal was also observed for several samples (Table 1). For most of the samples amplified with the 0.1μM concentration, analysis of the CE data could not be performed due to detector saturation.

Additional experiments were designed to further evaluate the use of the 0.1 μM primer concentration. Since detector saturation was observed for as little as 5 pg DNA, we evaluated the use of fewer pre-amplification cycles to determine if we could prevent detector saturation. The ability to recovery Y-STR alleles from 5 pg of male DNA would be a significant improvement compared to the sensitivity obtained by standard Y-STR analysis using the commercial kits. The use of 10 and 15 amplification cycles was examined and the results from this experiment can be found in Table 2. It was determined 15 cycles were needed for successful recovery of alleles from 5pg of input. In order to determine if a similar sensitivity would be observed with a different commercial Y-STR multiplex systems, the same 15-cycle pre-amplification samples were amplified with the Yfiler® multiplex. As can be seen from the results in Table 3, a similar 5 pg sensitivity was observed using Yfiler®. The results from this initial testing indicated that the

pre-amplification primers were suitable for use with either the Y-PLEX 12 or Yfiler® multiplex amplification kit (which use different primer sets) and similar sensitivity was achieved with both kits. For both multiplex systems, a significant improvement in sensitivity and allelic signal intensity was achieved for samples that had been pre-amplified using the nested PCR approach.

The previous experiments had involved the use of singleplex pre-amplification reactions. Since the same conditions were used for both loci, attempts were then made to create a duplex pre-amplification reaction. Both primer sets were incorporated into a single pre-amplification reaction. A range of input male DNA from 5 pg to 100 pg was used to evaluate the duplex pre-amplification. The previous experiments had demonstrated that using input amounts above 100 pg often resulted in detector saturation, so we focused on the lower input range. The results from the initial duplex testing can be seen in Table 4 and Figure 4. Alleles at both loci were detected using as little as 15 pg of input male DNA. The signal intensity of the obtained alleles were significantly higher than that obtained for the same input amount without prior pre-amplification. Indeed some alleles were not detected unless pre-amplification was used. This high sensitivity was observed with both the Y-PLEX™ 12 and Yfiler® multiplex systems. When 5 pg of input male DNA was used, only one of the two loci was detected. The same locus was amplified for both Y-STR multiplex systems, so it is likely that the second locus was not amplified in the pre-amplification reaction itself. However, these results indicate that the duplex pre-amplification reaction performs as well as the singleplex reactions and results in improved recovery of male DNA haplotypes with prior pre-amplification. We therefore focused on efforts to design suitable first round amplification primers for the remaining 15 commonly used Y-STR loci.

We evaluated alternative reaction mixtures to determine whether we could further improve the sensitivity of the pre-amplification method. Thus we evaluated a reaction mixture

that included a high fidelity non-Taq polymerase enzyme, Advantage® HD DNA polymerase (Clontech, Mountain View, CA). It permitted the use of 10-20 sec annealing and extension steps and therefore the pre-amplification reactions can be performed in ~1 hour. We also included a post-PCR purification of the pre-amplification products using MinElute columns to eliminate any unincorporated primers. At this stage, we had successfully designed suitable first round amplification primer sets for nine loci and therefore a 9-plex pre-amplification was developed using the Advantage® HD polymerase. Figure 5 shows the improvement in allele recovery and signal intensity when a 15 pg male DNA sample received prior pre-amplification. While the success of this pre-amplification method is evident, there was some inconsistency between amplifications. Periodically, a pre-amplification reaction would not provide consistent results in terms of allele recovery and improvement in signal intensity when using input amounts that had been previously successful (data not shown). It often took the use of a new tube of enzyme in order to again obtain the improved allele recovery previously observed. Therefore, it was determined that this method was not as consistent as the Type-It Microsatellite based pre-amplification and therefore all future experiments were performed using that amplification mix.

While extremely challenging and time consuming, we were finally able to design suitable first round amplification primers to allow for a multiplex pre-amplification of all 17-loci contained in the Yfiler® amplification system. It should be noted that 12 of these 17 loci are contained in the Promega Y-STR product, PowerPlex® Y System. While the primer sequences themselves are currently proprietary information, the size of the amplification products (~250 – 600 bp) and the final concentration in the pre-amplification multiplex are provided in Table 5. The size range for each locus is based on the number of alleles listed in STRbase. The DYS389 outer or first round amplification primers encompass both products and therefore only a single

amplification product is obtained unlike Y-STR typing multiplexes where two separate products are obtained.

To initially test the performance of the 17-locus nested PCR pre-amplification multiplex, we amplified male DNA (5, 10 and 15 pg from 10 donors) with and without pre-amplification and compared the number of alleles recovered and also the signal intensity of recovered alleles. The results of this initial study are provided in Table 6. Using 15 pg of male DNA, without prior pre-amplification, 3-5 alleles were obtained using standard RFU threshold values (>100 RFUs). If that threshold was reduced, additional loci could be detected although it is unlikely that operational crime laboratories would use a 20 RFU threshold. Using this same input amount, almost full profiles (15/17 alleles) were recovered if the samples received pre-amplification using the nested PCR multiplex. Using 10 pg of input, 14/17 alleles were recovered with prior pre-amplification compared to only 1-7 alleles recovered without pre-amplification. Using 5 pg of input (i.e. ~1 diploid cell), 10-11 alleles could be recovered with prior pre-amplification compared to only 1-5 alleles recovered without pre-amplification. Representative Yfiler® profiles from 5 pg samples amplified with and without prior pre-amplification are provided in Figure 6. The results of this study indicated that the developed nested PCR pre-amplification multiplex would be useful to improve the recovery of male DNA profiles from low template samples.

We tested the specificity of the nested PCR pre-amplification system using various amounts of female DNA (1ng, 100ng, 1000ng). Initially, no female DNA amplification products were observed. However, throughout the subsequent optimization and validation experiments, one or two peaks were observed for female samples on the blue channel just outside of the DYS456 allele range (Figure 7). These peaks sometimes were present and then were absent in other samples and then signal intensity ranged from barely above the detection threshold to quite

high. It is unclear what these amplification products are, but are likely the result of a partial homology of one or more of the primer sets in the pre-amplification multiplex. However, they are not located within a Y-STR locus allele range and therefore are not considered critical. It is possible that amplification of these products could result in less efficient amplification of male DNA (i.e. titration of critical PCR reagents since female DNA would be found in significant excess of the male DNA in the sample). However, throughout the validation experiments (see below), particularly the mixture studies, there did not appear to be a noticeable decrease in amplification efficiency when female DNA was present.

Validation of the Y-Chromosome Specific Nested PCR Pre-Amplification Assay

After the development and successful initial testing of the developed pre-amplification multiplex, we then proceeded to validate the pre-amplification multiplex. The validation experiments included: 1) Y-STR kit compatibility, 2) body fluid and tissue studies; 3) mixtures (male/female, male/male, male/male/female, male/male/male), 4) mock case work samples (laser capture micro-dissected cells, touch DNA, extended interval post coital samples); and 5) environmentally compromised samples.

(1) Compatibility with Commercially Available Y-STR Kits - Previous experiments were conducted using Yfiler®. We needed to check that the pre-amplification multiplex was also compatible with PowerPlex® Y (Promega). For this study, 5 – 15 pg of male DNA from three different donors was pre-amplified and then aliquots of the pre-amplification product were amplified with the PowerPlex® Y kit. The results are provided in Table 7. Allele recovery is indicated using several different RFU thresholds (20, 50, 100 and 150 RFUs) since operational crime laboratories might utilize different thresholds. As can be seen from these results, there was

improved allele recovery for the PowerPlex® Y multiplex as well an increase in the allelic signal intensity for detected alleles. The number of alleles recovered provided in Table 7 represent an average of the three male profiles and does not indicate which, if any, were full male profiles. A full male profile was obtained for the 10pg samples for male 1 and male 2. The full profile recovered from one of the males after pre-amplification is shown in Figure 8A. The presence of two alleles (30 and 31) was observed for DYS389II (Figure 8A). The 31 allele is the correct allele for that donor and therefore the 30 allele could be an increased stutter or possible drop-in allele. Before implementation into casework, laboratories should further investigate stutter ratios in order to determine if, and under what conditions, increased stutter can be observed. However, high stutter peaks were not frequently observed throughout the validation exercises despite the low template nature of most of the samples

In addition to the male profiles, we also amplified a female DNA sample (263ng input). No amplification products were observed with this input amount (Figure 8B). While only a small number of samples were evaluated in this study, it was evident that both the PowerPlex® Y and Yfiler® multiplex kits could be used for subsequent Y-STR analysis of pre-amplified samples. We primarily use Yfiler® in our laboratory (due to the increased number of loci) and therefore the remaining validation studies were performed using Yfiler®.

(2) Body Fluid and Tissue Studies - The samples used in the previous experiments described were extracted from dried bloodstains. We also tested blood, saliva, semen and skin samples from a male donor and used 5 pg, 10 pg and 15 pg of extracted DNA from these body fluids/tissues in the pre-amplification multiplex. We also amplified the same input amounts directly without pre-amplification. The results from this study are provided in Table 8. Using a 100 or 150 RFU threshold, the use of pre-amplification resulted in an increase in the number of

alleles obtained as well as a significant increase in allelic signal intensity. When a lower threshold was used (20 or 50 RFUs), in most cases an increase in the number of alleles was obtained (the only exceptions being blood (5 pg) and semen (10 pg) where one additional allele was observed without pre-amplification).

We found some inconsistency in allele recovery amongst the various body fluids from the same donor using 5 pg and 10 pg input amounts (Table 8). Fewer alleles were recovered from semen compared to blood and saliva, which was a concern since the forensic cases in which the pre-amplification multiplex would be particularly useful would involve sexual assault. We tested semen samples from other individuals to determine if pre-amplification of semen DNA was going to be less efficient than the other body fluids (data not shown). We extracted samples using both an organic extraction and the DNA Investigator kit (QIAGEN) in order to determine if there were extraction efficacy problems with semen compared to the other body fluids. Using an organic extraction, an increased number of alleles were observed for the 10 pg input level (data not shown); however there were still few alleles recovered at the 5 pg level. Interestingly, an increased number of alleles was observed when the DNA Investigator kit was used for extraction (data not shown). Despite the variable success observed here, during our evaluation of mock casework samples, including post coital samples, an improvement in profile recovery was observed. The analysis of these samples included an extract concentration and purification step prior to pre-amplification, which could account for the improvement in profile recovery observed. The concentration/purification step was subsequently used as part of the standard protocol.

(3) Mixtures: Two Donor Mixtures (Male/Female) - We next evaluated the ability to recover male profiles in male/female mixtures in which the female DNA was in excess. For this study,

we amplified 5, 10 and 15 pg of male DNA with increasing amounts of female DNA (5 pg to 1800 ng, mixture ratios of 1:1 to 1:368,000) (Table 9). Five male donors were used for each input amount and the averaged data is provided (Table 9). In each mixture, 9-13 male alleles (out of 17) were recovered when the mixtures were pre-amplified with the nested PCR pre-amplification multiplex. Without this pre-amplification, only 2-5 male alleles were obtained using a 100-150 RFU threshold. Additional alleles were observed when lower thresholds were used 20-50 RFU); these are lower than that used by most crime labs. With pre-amplification, the same number of alleles were recovered using any of the four thresholds, indicating the dramatically increased signal intensity of these alleles compared to the same sample without pre-amplification. While only partial profiles were obtained for a majority of these samples, increasing amounts of female DNA did not result in a loss of alleles and therefore indicates that female DNA (in significant excess) does not interfere with the ability to improve the recovery of male DNA profiles. For all mixture studies, male DNA of the same input was also amplified without the addition of female DNA for comparison to the mixture studies. There was no loss of allele recovery with the addition of female DNA the male DNA in any of the mixture studies performed.

The 5 pg input (~1 diploid cell) produces partial profiles with pre-amplification. Therefore we tried additional inputs (10 and 15 pg) to ascertain whether more complete profiles would be obtained. The same five males were used for these mixtures as well. For all mixture amounts using a 10 pg input, 13 – 14 male alleles were recovered from the samples that were pre-amplified compared to only 3 – 6 alleles without (Table 9). Using 15 pg of input, nearly full profiles were obtained for all samples that were pre-amplified (15-16 alleles out of 17) with only

4 – 9 alleles without. (Table 9). In all cases, no reduction in allele recovery was observed with increased amounts of female DNA.

Two Donor Mixtures (Male/Male) –Male-male DNA mixtures, using 1:1 ratios with 5, 10 and 15 pg of input were tested. Two sets of two-donor mixtures (four separate donors) were prepared and averages of the RFU values and number of alleles recovered were made from the two sets. The results of the male-male mixtures are provided in Table 10. With prior pre-amplification, consistent numbers of alleles were recovered for both males in the mixture. In each case, fewer alleles and significantly reduced signal intensities were observed for samples that did not receive prior pre-amplification.

Three Donor Mixtures (Male/Male/Female) - We prepared male-male-female and male-male-male mixtures. This was a very small study conducted only as a proof of concept, not for any in depth mixture analysis. The results from the three donor mixtures are provided in Table 11 (male-male-female) and table 12 (male-male-male). In all multiple donor mixtures tested, we were able to obtain similar numbers of alleles from all male donors in the study (Tables 11 and 12).

These mixture studies were for proof-of-concept only and the results should be replicated using more samples and different mixture ratios.

(4) Mock Casework Samples -

In other work, we determined that the use of a DNA purification and concentration step prior to downstream PCR analysis resulted in improved profile recovery. We therefore evaluated

its use with the remaining validation studies, specifically the mock casework (described here) and environmentally compromised samples (shown below). Samples were extracted using a standard organic extraction and re-solubilized in 75 - 100 TE^{-4}. The entire extract was then purified using MinElute spin filtration columns (QIAGEN) and eluted into 12 µl of nuclease free water. The use of the reduced elution volume permitted the addition of most of the entire extract (7.5 µl) to be added to the pre-amplification reaction. All experiments described below (with the exception of LCM samples) utilized this additional step. The purification/concentration step is now part of the standard protocol.

Mock Casework (Laser Capture Micro-Dissection) - We collected varying numbers of buccal epithelial cells (1, 2, 3 5 and 10 cells) from four different male donors using laser capture micro-dissection (Leica ASLMD system). The Leica ASLMD system uses a laser to cut around the targeted cells of interest, which then fall by gravity into the cap of a 0.2ml PCR tube. Direct lysis and PCR can be performed in the same tube. A direct lysis protocol was performed on these samples (unpublished method which will be described in a separate publication). Since a direct lysis reaction was used, no purification of the samples was performed before amplification with the pre-amplification multiplex. The lysates could therefore contain inhibitors that could affect amplification efficiency. The results from the LCM samples are provided in Table 13. Without prior pre-amplification, few alleles, if any, were detected for the 1 -5 cell samples (Table 13). A small number of alleles were obtained for the 10-cell sample, although with extremely low peak heights (Table 13). However, when the samples were pre-amplified, we were able to recover an increased number of alleles: 5 – 13 alleles with significantly higher signal intensities. For the 1-cell samples, the average number of alleles recovered could be somewhat misleading due to the

fact that no profile was obtained for two of the four donors. Partial profiles, with 10 and 11 alleles (out of 17) recovered, were obtained for the other two donors. The profile recovered from one of these 1-cell samples is shown in Figure 9. Without pre-amplification, only one low-intensity allele was observed, but with pre-amplification 11/17 alleles were observed all with high signal intensity (Figure 9). The ability to obtain potentially probative partial profiles from even a single buccal cell dramatically demonstrate the potential efficacy of the nested pre-amplification method for casework.

Mock Casework (Touch DNA Samples) –We tested swabs taken from contacted or handled objects and surfaces including a door handle (single occupant office), a coffee cup, a phone handle and a water jug lid. The coffee cup, phone handle and water jug were associated with a particular office area and would therefore likely contain DNA from the same individual. All samples were extracted using a standard organic extraction and were then concentrated and purified using the MinElute columns as described above. Five microliters of the twelve microliter extract was used for pre-amplification and another five was used directly in the Yfiler® amplification for comparison. Table 14 provides the results for each of the four samples. As can be seen from this data, no alleles (with the exception of the 13 alleles obtained for DYS393) were obtained without prior pre-amplification (Table 13). When these same samples were amplified with the pre-amplification multiplex prior to Y-STR amplification, full profiles were obtained for all samples, except for the water jug lid sample in which 16/17 alleles were obtained. For those samples where alleles were recovered without pre-amplification, there was a significant increase in not only the number of alleles recovered but also the signal intensity of those alleles. Therefore, while this impetus for this study was to develop methodologies for the

analysis of sexual assault samples, the pre-amplification method is suitable for other sample types involving low template amounts of male DNA, including touch DNA samples.

Mock Casework (Post Coital Samples) – Four donor couples were recruited for this study. Post-coital cervicovaginal swabs (x2) were recovered by each of the four females at specified intervals after sexual intercourse (6 – 9 days). Each time point sample was collected after a separate act of sexual intercourse. Donor couples were asked to abstain from sexual intercourse for an additional 2-3 days after previous sample collection in order to provide an overall 8-12 day period in between sexual intercourse in order to reduce the potential for residual semen to be present before starting the collection process for the next time interval. With the exception of couple 1, all donor couples collected a pre-coital swab prior to coitus for each sampling as a control to demonstrate the amount, if any, of residual semen from a previous sexual act. No male alleles were detected in the pre-coital swabs for couples 2 and 3 (6 and 7 day time points) (data not shown). A small number of alleles matching the profile of the male participant were detected in the pre-coital swabs from couple 4 for the 7, 8 and 9 day samples (3, 2 and 1 allele respectively) after the enhanced profiling techniques described below (data not shown). The presence of these alleles did not preclude the use of these samples in subsequent studies. Donor couples were asked to abstain from sexual intercourse only and therefore trace amounts of male DNA may have been present from other forms of sexual contact. Additionally, based on our previous work, it was assumed that a ~ 8 day abstention period was sufficient since we only obtained profiles routinely out to 5 – 6 days [35]

One of the two swabs collected at each time point was extracted using a non-differential organic extraction. The remaining swab from each time point was stored for possible further analysis, with the exception of the 9 day samples for couples 1 and 4 in which both swabs were

extracted for comparison (described below). The extracted DNA was re-solubilized in 75 µl of TE^{-4}, purified with the MinElute kit (QIAGEN) and eluted into 12 µl of nuclease free water to use for quantitation (2 µl), nested PCR pre-amplification (5 µl), and a non-pre-amplification control in subsequent Y-STR analysis (5 µl). While these aliquots were required for this study, non-pre-amplification controls would not be used in casework and therefore this aliquot could be reserved for additional testing if needed.

All samples were quantitated using the Quantifiler® Y Human Male DNA quantification kit (AB by Life Technologies). However, undetectable quantities were observed for all samples and although this quantitation step could be eliminated in order to improve timeliness and reduce sample consumption, such a procedure would need an appropriate change to the National Quality Assurance Standards for DNA Testing Laboratories. The 5 microliter aliquots of purified and concentrated extract were used in a subsequent Y-chromosome specific nested PCR pre-amplification (25 µl reaction volume). The pre-amplification PCR products were then purified using the MinElute PCR purification kit (QIAGEN) with an elution volume of 25 µl (nuclease free water). A 0.5 µl aliquot of the purified pre-amplification product was used in a subsequent amplification using the AmpFlSTR® Yfiler® PCR amplification kit (AB by Life Technologies) (12.5 µl reaction volume). Five microliter aliquots of the non-pre-amplified extract were included in this amplification for comparison.

The typing results of the post coital samples subjected to a non-differential extraction, followed by extract purification and concentration, are summarized in Table 15. Representative electropherograms from an 8- and 9- day post coital sample are shown in Figures 11 and 12, respectively. For each sample, two electropherograms are shown: A) without pre-amplification and b) with pre-amplification. For a majority of samples no alleles were detected without the use

of the pre-amplification. However, it was interesting to note that a small number of alleles were present in a few non-pre-amplified samples (couple 1 – 6 day (1), couple 2 – 7 day (4), and couple 4 – 8 day (4)) (Table 15, Figure 11A). During initial experiments in the early stages of this work prior to the development of the enhanced profiling strategies, no alleles were obtained at this collection time points using standard extraction protocols (data not shown). These protocols utilized small aliquots from a larger extract volume (5 - 10 μl from a 50 - 100 μl extract). While this is by far not the first study to utilize sample purification or concentration, we demonstrate here its beneficial use in the analysis of extended interval post coital samples as a simple additional procedural step that on its own permitted the recovery of male donor alleles. It was a hopeful indication that additional modifications would serve to further improve profile recovery in these extended interval post coital samples.

As can be seen from Table 15, the use of the Y-chromosome specific nested PCR pre-amplification resulted in a significant increase in the number of alleles detected, with the recovery of ≥70% of the male donor alleles (with the exception of couple 3) (Table 15). Remarkably, full or nearly full profiles were even obtained from samples collected 8 and 9 days after intercourse (couples 1 and 4) (Table 15, Figures 11 and 12). To our knowledge this is the longest ever post-coital time interval from which a genetic profile of the semen donor has been recovered. Our own previous work led to the routine recovery of male donor profiles from samples collected only up to 5 days, with only limited partial profiles obtained for 6 days and a single 7-day sample.

The use of the Y-chromosome specific nested PCR pre-amplification, therefore, significantly improved the time interval in which the profile of the semen donor can be obtained. With the strength of the profiles recovered from the 9-day samples for couples 1 and 4, it is

possible that we have not yet reached the limits of detection. Additional samples beyond the 9-day period will be collected and evaluated in future studies.

While we obtained successful results for a majority of samples in this study, it is expected that not all extended interval post-coital samples will be successful. A variety of factors will influence the amount of sperm and/or male epithelial cell remaining in the female victim such as (but not limited to): 1) activity level of the victim after assault; 2) victim showering, bathing, douching after assault; 3) occurrence of the assault during the victim's menstruation cycle; 4) sperm count of the perpetrator; 5) volume of semen ejaculated during the assault; and 6) number of times of ejaculation during the assault. These factors will all contribute to the amount of male DNA available for collection and analysis. The potential differential success in profile recovery from samples collected from different individuals within the same time interval is evident even in this study with only four donor couples, with significantly less alleles recovered for couple 3 (6 day – 24% of alleles; 7 day – 18% of alleles) (Table 15). Additionally, the samples in this study were self-collected and it is therefore possible that some donors experienced greater difficulty with sample collection thereby affecting the amount of biological material recovered on those samples. While there are many variables affecting successful profile recovery, the results of this study indicate that the novel enhanced profiling strategies developed here should provide an improved potential for profile recovery.

(5) Environmentally Compromised Samples – Due to the large size of the pre-amplification multiplex products, there was some concern that the system may not be suitable for use with highly degraded or environmentally compromised samples. In order to test this hypothesis, we pre-amplified DNA from environmentally compromised blood samples (exposed to 37^{o}C or

56°C or stored outside exposed to heat, light, humidity and rain). Since this essentially was a preliminary proof-of-concept study on the effects of DNA quality on pre-amplification efficacy, we evaluated the latest time point available for each of those conditions (37°C and 56°C – 2 year exposure; outside covered and uncovered – 6 months exposure). The results from this study are provided in Table 16. Without prior pre-amplification a small number of alleles were observed for all samples except the sample stored outside uncovered (exposed to heat, light humidity, and rain) (Table 16). When the same samples were pre-amplified, an increase in the number of alleles recovered and the signal intensity of those alleles were improved. Surprisingly, a full profile was obtained by pre-amplification for the bloodstain stored outside covered (exposed to heat, light and humidity for 6 months). The results of this study indicate that it may be possible to recover some genetic information from degraded samples, but that variable success would be achieved depending on the extent of the degradation. Further studies will be needed in order to fully evaluate the use of the pre-amplification multiplex with degraded and compromised samples.

Strategies for the Amplification of Degraded Y Chromosomal DNA

At the commencement of this project, Qiagen had recently established proprietary technologies for the amplification of damaged total genomic DNA in a two-step process: a processing reaction preparing damaged DNA for whole genome amplification and an amplification reaction (unpublished observations). The original intent was to therefore utilize novel proprietary technology to attain specific restoration of amplifiable Y chromosomal DNA from degraded DNA. However, since the whole genome amplification based approaches using MDA was not successful, we were not able to utilize this technology.

Since an evaluation of potential restoration methods for use with degraded DNA was an aim of the current project, Bond University performed various studies in order to develop such methods. This work involved determining the conditions for generating samples with different levels of degradation in a reproducible manner and the development of an assay for determination of the quality and quantity of the degraded DNA [36]. This assay is a multiplex real-time quantitative PCR (qPCR) assay, which simultaneously quantifies total human genomic DNA, male DNA, the extent of DNA degradation and the presence of PCR inhibitors and was a modification of the Hudlow et al, 2008 qPCR assay. This assay may prove useful in future experiments involving degraded or highly compromised samples in order to assess the extent of degradation prior to the use of the pre-amplification multiplex.

Experiments were also performed to evaluate various DNA repair methods in an effort to improve DNA typing success. The approaches evaluated include the use of T4 ligase (+/- WGA), T4 + CircLigase + WGA, CicLigase + WGA, Restorase and PreCR. Despite the efforts that were made to develop these methods, none of these treatment resulted in any significant improvement. The Bond University final report is included in Appendix A and contains full details of the experiments that were performed.

B. TABLES

Table 1. Evaluation of the Initial Singleplex Nested PCR Pre-Amplification (20 cycles)

	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6	Mix 7	Mix 8
locus	393	393	393	393	391	391	391	391
final primer conc	0.1μM	0.1μM	0.01μM	0.01μM	0.1μM	0.1μM	0.01μM	0.01μM
Q-solution added	no	yes	no	yes	no	yes	no	yes
4124 - 1ng	SAT.	SAT.	**13**	**13**	SAT.	SAT.	**11**	**11**
RFU			5310	1755			7946	7004
4124 - 500 pg	SAT.	SAT.	**13**	**13**	SAT.	SAT.	**11**	**11**
RFU			3291	1371			7849	1788
4124-50 pg	SAT.	**13**	**13**	ND	SAT.	SAT.	**11**	**11**
RFU		7672	1672				1053	522
4124 - 5pg	SAT.	**13**	ND	ND	SAT.	ND	**11**	ND
RFU		7438					230	
Blank	ND	ND	ND	ND	ND	ND	ND	ND
Female-100ng	SAT.	SAT.	SAT.	X	SAT.	SAT.	SAT.	SAT.
RFU				7821				
	Without pre-amp							
	393 (13)				**391 (11)**			
4124 - 1ng	**13**				**11**			
RFU	1835				1128			
4124 - 500pg	**13**				**11**			
RFU	691				370			
4124 - 50 pg	**13**				**ND**			
RFU	175							
4124 - 5pg	**ND**				**ND**			
RFU								

*SAT = saturated

Table 2. Evaluation of the Initial Singleplex Nested PCR Pre-Amplification Using Reduced Cycles

10 cycles				
	Mix 1	**Mix 2**	**Mix 3**	**Mix 4**
locus	393	393	391	391
final primer conc	0.1μM	0.1μM	0.1μM	0.1μM
Q-solution added	no	yes	no	yes
4124 - 100 pg	**13**	**13**	**11**	**11**
RFU	1902	1069	1960	2177
4124 - 50 pg	**13**	**13**	**11**	**11**
RFU	1080	436	1514	965
4124-25 pg	**13**	**13**	**11**	**11**
RFU	527	393	1177	330
4124 - 15 pg	**13**	**ND**	**11**	**11**
RFU	244		197	315
4124 - 5 pg	**ND**	**13**	**ND**	**ND**
RFU		187		
Blank	ND	ND	ND	ND
Female-10ng	13, X	X	X	X
RFU	294, 3655	3322	3446	3251
	Without pre-amp			
	393 (13)		**391 (11)**	
4124 - 100pg	**13**		**11**	
RFU	77		116	
4124 - 50pg	**13**		**11**	
RFU	107		28	
4124 - 25pg	**ND**		**11**	
RFU			38	
4124 - 15pg	**ND**		**11**	
RFU			19	
4124 - 5pg	**ND**		**ND**	
RFU				
9948 CONTROL	**13**		**11**	
RFU	2270		1539	

15 cycles				
	Mix 1	**Mix 2**	**Mix 3**	**Mix 4**
locus	393	393	391	301
final primer conc	0.1μM	0.1μM	0.1μM	0.1μM
Q-solution added	no	yes	no	yes
4124 - 100 pg	**13**	**13**	**11**	**SAT.**
RFU	6926	7598	7790	
4124 - 50 pg	**SAT.**	**13**	**11**	**11**
RFU		5073	7835	7024
4124-25 pg	**13**	**13**	**ND**	**11**
RFU	7774	3961		7116
4124 - 15 pg	**ND**	**13**	**11**	**11**
RFU		1556	7786	4665
4124 - 5 pg	**13**	**ND**	**11**	**11**
RFU	6437		7817	1666
Blank	ND	ND	ND	ND
Female-10ng	SAT.	13, X	X	X
RFU		1279, 1628	2828	2596
	Without pre-amp			
4124 - 100pg	**13**		**11**	
RFU	116		357	
4124 - 50pg	**13**		**11**	
RFU	75		165	
4124 - 25pg	**ND**		**ND**	
RFU				
4124 - 15pg	**ND**		**ND**	
RFU				
4124 - 5pg	**ND**		**ND**	
RFU				
9948 CONTROL	**13**		**11**	
RFU	4095		3246	

Table 3. Comparison of the Singleplex Nested PCR Pre-Amplification Using Two Different Y-STR multiplex systems

Yplex 12 - 1µl of pre-amp product added to amp; 1µl on 3130				
Pre-amp: 95°C 15 min; 15 cycles 95°C 30 sec, 60°C 90 sec, 72°C 30sec; 68°C 10 min; 8°C hold				
	Mix 1	Mix 2	Mix 3	Mix 4
locus	393	393	391	391
final primer conc	0.1µM	0.1µM	0.1µM	0.1µM
Q-solution added	no	yes	no	yes
4124 - 100 pg	13	13	11	SAT.
RFU	6926	7598	7790	
4124 - 50 pg	SAT.	13	11	11
RFU		5073	7835	7024
4124-25 pg	13	13	ND	11
RFU	7774	3961		7116
4124 - 15 pg	ND	13	11	11
RFU		1556	7786	4665
4124 - 5 pg	13	ND	11	11
RFU	6437		7817	1666
Blank	ND	ND	ND	ND
Female-10ng	SAT.	13, X	X	X
RFU		1279, 1628	2828	2596
	Without pre-amp			
	393 (13)		391 (11)	
4124 - 100pg	13		11	
RFU	116		357	
4124 - 50pg	13		11	
RFU	75		165	
4124 - 25pg	ND		ND	
RFU				
4124 - 15pg	ND		ND	
RFU				
4124 - 5pg	ND		ND	
RFU				
9948 CONTROL	13		11	
RFU	4095		3246	

Yfiler - 0.5µl of pre-amp product added to amp; 0.5µl on 3130				
Pre-amp: 95°C 15 min; 15 cycles 95°C 30 sec, 60°C 90 sec, 72°C 30sec; 68°C 10 min; 8°C hold				
	Mix 1	Mix 2	Mix 3	Mix 4
locus	393	393	391	391
final primer conc	0.1µM	0.1µM	0.1µM	0.1µM
Q-solution added	no	yes	no	yes
4124 - 100 pg	SAT.	SAT.	11	11
RFU			8280	7852
4124 - 50 pg	13	13	11	11
RFU	6602	7388	7885	8294
4124-25 pg	13	13	ND	11
RFU	8008	7461		6935
4124 - 15 pg	ND	SAT.	11	11
RFU			7247	7936
4124 - 5 pg	13	ND	11	11
RFU	8043		8096	2419
Blank	ND	ND	ND	ND
Female-10ng	OL PEAKS	OL PEAKS	OL PEAKS	OL PEAKS
RFU				
	Without pre-amp			
4124 - 100pg	13		11	
RFU	420		1173	
4124 - 50pg	13		11	
RFU	175		1031	
4124 - 25pg	13		11	
RFU	249		262	
4124 - 15pg	13		11	
RFU	104		105	
4124 - 5pg	ND		11	
RFU			122	
007 CONTROL	13		11	
RFU	1589		3636	

Table 4. Comparison of the Duplex Nested PCR Pre-Amplification Using Two Different Y-STR multiplex systems

Yplex 12 - 1µl of pre-amp product added to amp; 1µl on 3130				
Pre-amp: 95°C 15 min; 15 cycles 95°C 30 sec, 60°C 90 sec, 72°C 30sec; 68°C 10 min; 8°C hold				
	Mix 1		Mix 2	
locus	393	391	393	391
final primer conc	0.1µM		0.1µM	
Q-solution added	no		yes	
4124 - 100 pg	13	11	13	11
RFU	7716	7325	7985	7948
4124 - 50 pg	13	11	13	11
RFU	7784	7987	5241	7078
4124-25 pg	13	11	13	11
RFU	4228	7571	7994	8029
4124 - 15 pg	13	11	13	11
RFU	7710	196	4278	6645
4124 - 5 pg	13	ND	ND	11
RFU	1907			7615
Blank	ND			
Female-10ng	12, 13, 15	X	12, 13, 15	X
RFU	2161, 4854, 2144	2865	778, 1581, 662	2031
	Without pre-amp			
	393 (13)		391 (11)	
4124 - 100pg	13		ND	
RFU	192			
4124 - 50pg	ND		ND	
RFU				
4124 - 25pg	ND		ND	
RFU				
4124 - 15pg	ND		ND	
RFU				
4124 - 5pg	ND		ND	
RFU				
9948 CONTROL	13		11	
RFU	4578		3997	

Yfiler - 0.25µl of pre-amp product added to amp; 0.5µl on 3130				
Pre-amp: 95°C 15 min; 15 cycles 95°C 30 sec, 60°C 90 sec, 72°C 30sec; 68°C 10 min; 8°C hold				
	Mix 1		Mix 2	
locus	393	391	393	391
final primer conc	0.1µM		0.1µM	
Q-solution added	no		yes	
4124 - 100 pg	13	11	13	11
RFU	7212	8080	7728	8098
4124 - 50 pg	13	11	13	11
RFU	7436	8214	8409	8237
4124-25 pg	13	11	13	11
RFU	8810	8181	7880	8584
4124 - 15 pg	13	11	13	11
RFU	8250	551	4837	8991
4124 - 5 pg	13	ND	ND	11
RFU	5696			8565
Blank	ND		ND	
Female-10ng	DYS19-13?	OL peaks	DYS19-13?	
RFU	500	<400	66	
	Without pre-amp			
	393 (13)		391 (11)	
4124 - 100pg	3130 ISSUE - DIDN'T ANALYZE		3130 ISSUE - DIDN'T ANALYZE	
RFU				
4124 - 50pg	13		11	
RFU	328		419	
4124 - 25pg	13		11	
RFU	75		93	
4124 - 15pg	ND		11	
RFU			139	
4124 - 5pg	13		ND	
RFU	56			
007 CONTROL	13		11	
RFU	1538		1466	

Table 5. Characteristics of the 17-plex Nested PCR Pre-Amplification

Locus	Pre-Amplification Product Size (bp)	Reference Allele	Size Range (bp)	Final Primer Conc (μM)
DYS19	301	16	273 - 313	0.35
DYS385	510	17	470 - 554	0.15
DYS389	576	12, 29	556 - 596	0.20
DYS390	405	29	361 - 405	0.60
DYS391	418	14	386 - 418	0.06
DYS392	596	13	575 - 608	0.12
DYS393	422	12	410 - 442	0.05
DYS437	270	16	258 - 274	0.04
DYS438	386	10	366 - 406	0.06
DYS439	373	13	357 - 377	0.60
DYS448	534	19	534 - 576	0.13
DYS456	337	14	333 - 353	0.10
DYS458	423	16	411 - 439	0.04
DYS635	503	23	479 - 515	0.20
H4	414	10	406 - 426	0.14

Table 6. Sensitivity of the 17-plex Nested PCR Pre-Amplification Multiplex

Input	Pre-amp?	Avg RFU	Avg # Loci			
			>150	>100	>50	>20
15pg	with	4088	15	15	15	15
	without	105	3	5	9	11
10pg	with	3727	14	14	14	14
	without	91	1	3	7	9
5pg	with	4272	10	10	11	11
	without	89	1	2	5	6

*Averages based on data from 10 male individuals

Table 7. Comptability of Pre-Amplification Multiplex with Commercially Available Y-STR kits (Yfiler® and PowerPlex® Y)

		Yfiler					PowerPlex Y				
Input	Pre-amp?	Avg RFU	Avg # Loci (17 possible)				Avg RFU	Avg # Loci (12 possible)			
			>150	>100	>50	>20		>150	>100	>50	>20
15pg	with	4088	15	15	15	15	6045	10	10	10	10
	without	105	3	5	9	11	402	8	9	10	10
10pg	with	3727	14	14	14	14	5756	11	11	11	11
	without	91	1	3	7	9	172	5	7	8	10
5pg	with	4272	10	10	11	11	5778	7	7	7	7
	without	89	1	2	5	6	114	1	2	5	5
			avgerage of 10 males 30 cycles - std 25ul MinElute					average of 3 males 32 cycles - std 50ul MinElute			

Table 8. Somatic Stability: Recovery of Y-STR profiles from Multiple Body fluids and tissues from the Same Donor

Body Fluid/tisue	Input	Pre-amp?	Avg RFU	Avg # Loci			
				>150	>100	>50	>20
Blood	5pg	with	4853	15	16	16	16
		without	213	13	13	17	17
	10pg	with	4085	17	17	17	17
		without	253	13	16	17	17
	15pg	with	8145	17	17	17	17
		without	336	17	17	17	17
Semen	5pg	with	7395	7	7	7	7
		without	91	1	2	4	6
	10pg	with	7749	8	8	8	8
		without	117	5	7	9	13
	15pg	with	5547	17	17	17	17
		without	155	7	10	12	14
Saliva	5pg	with	6871	6	6	6	6
		without	65	0	0	4	5
	10pg	with	5015	4	4	4	4
		without	41	0	0	0	3
	15pg	with	3231	12	12	12	12
		without	83	0	3	5	8
Skin Swab	5pg	with	NA	0	0	0	0
		without	NA	0	0	0	0
	10pg	with	1459	2	2	2	2
		without	69	0	0	1	1
	15pg	with	NA	0	0	0	0
		without	64	0	1	1	4
Skin Swab (undiluted)	1µl	with	6678	14	14	14	14
		without	95	2	4	8	11
	2µl	with	4768	17	17	17	17
		without	217	7	14	16	16
	5µl	with	6535	17	17	17	17
		without	602	14	17	17	17

*Same male individual for all fluids/tissues

Table 9. Ability to Obtain Male Y-STR Profiles from Male/Female DNA Admixtures with Increasing Amounts of Female DNA

5pg								
Mixture	Male DNA	Female DNA	Pre-amp?	Avg RFU	Avg # Loci			
					>150	>100	>50	>20
1:1	5pg	5pg	with	5813	9	9	9	9
			without	113	2	5	9	9
1:10	5pg	50pg	with	4865	10	10	10	10
			without	118	2	4	8	9
1:100	5pg	500pg	with	7018	9	9	9	9
			without	111	2	4	8	9
1:1,000	5pg	5ng	with	6795	9	9	9	9
			without	119	2	3	9	10
1:10,000	5pg	50ng	with	6051	10	10	10	10
			without	118	2	3	8	8
1:100,000	5pg	500ng	with	5655	13	13	13	13
			without	94	1	1	3	4
1:368,200	5pg	1841ng	with	7074	9	9	9	9
			without	NA	NA	NA	NA	NA
NA	5pg	NA	with	6664	11	11	11	11
			without	101	2	4	7	9

10pg								
Mixture	Male DNA	Female DNA	Pre-amp?	Avg RFU	Avg # Loci			
					>150	>100	>50	>20
1:1	10pg	10pg	with	4913	14	14	14	14
			without	114	3	5	9	11
1:10	10pg	100pg	with	4675	13	13	13	13
			without	105	3	5	11	13
1:100	10pg	1ng	with	5633	14	14	14	14
			without	130	4	6	12	13
1:1,000	10pg	10ng	with	6540	14	14	14	14
			without	116	3	6	11	12
1:10,000	10pg	100ng	with	6286	14	14	14	14
			without	112	3	5	9	11
1:100,000	10pg	1000ng	with	5942	14	14	14	14
			without	88	1	2	5	6
1:170,900	10pg	1709ng	with	5485	13	13	13	13
			without	NA	NA	NA	NA	NA
NA	10pg	NA	with	6064	14	14	14	14
			without	130	3	6	10	11

15pg								
Mixture	Male DNA	Female DNA	Pre-amp?	Avg RFU	Avg # Loci			
					>150	>100	>50	>20
1:1	15pg	15pg	with	5851	16	16	16	16
			without	168	5	9	14	15
1:10	15pg	150pg	with	6224	16	16	16	16
			without	163	6	8	12	14
1:100	15pg	1.5ng	with	5617	15	15	15	15
			without	135	4	7	10	10
1:1,000	15pg	15ng	with	6313	15	15	15	15
			without	134	4	8	12	13
1:10,000	15pg	150ng	with	5828	15	15	15	15
			without	155	5	8	13	15
1:100,000	15pg	1500ng	with	5556	16	16	16	16
			without	NA	NA	NA	NA	NA
1:105,200	15pg	1578ng	with	5603	15	15	15	15
			without	NA	NA	NA	NA	NA
NA	15pg	NA	with	6002	15	15	15	15
			without	158	6	8	12	12

*The NA for designations for the “without” sample for the highest female input amounts indicates that that volume of female DNA could not be added to the Yfiler amplification since only a ½ reaction volume was used.

Table 10. Improved Profile Recovery of Male Profiles in Two Male Donor Mixture After Pre-Amplification

		Avg RFU		Avg # Loci (17 possible)							
Input	Pre-amp?	Male 1	Male 2	>150		>100		>50		>20	
				Male 1	Male 2	Male 1	Male 2	Male 1	Male 2	Male 1	Male 2
5pg	with	4819	4994	12	12	12	12	12	12	12	12
	without	95	135	2	5	5	7	9	11	12	12
10pg	with	4281	4301	16	16	16	16	16	16	16	16
	without	195	175	4	7	7	10	11	13	12	13
15pg	with	3828	3139	15	17	15	17	15	17	15	17
	without	195	204	8	9	9	11	14	15	15	17

*Averages based on two 2-male mixture sets (four donors total)

Table 11. Improved Profile Recovery of Male Profiles in Three Donors Mixtures (Male-Male-Female) After Pre-Amplification

				Avg RFU		Avg # Loci (17 possible)							
Male input	Female input	Ratio	Pre-amp?	Male 1	Male 2	>150		>100		>50		>20	
						Male 1	Male 2	Male 1	Male 2	Male 1	Male 2	Male 1	Male 2
5pg	1052ng	1:210,400	with	4282	3827	7	13	7	13	7	13	7	13
			without	70	67	0	0	1	1	5	7	7	8
10pg	789ng	1:78,900	with	3551	3094	11	17	11	17	11	17	11	17
			without	119	132	2	3	4	7	7	10	9	12

*Averages based on two 2-male mixture sets (four donors total)

Table 12. Improved Profile Recovery of Male Profiles in Three Male Donor Mixtures After Pre-Amplification

		Avg RFU			Avg # Loci (17 possible)											
Input	Pre-amp?	Male 1	Male 2	Male 3	>150			>100			>50			>20		
					Male 1	Male 2	Male 3	Male 1	Male 2	Male 3	Male 1	Male 2	Male 3	Male 1	Male 2	Male 3
5pg	with	3363	4727	5155	11	14	9	11	14	9	11	14	9	11	14	9
	without	77	92	81	0	1	0	1	4	2	7	9	8	8	11	10
10pg	with	4634	5204	5177	13	14	10	13	14	10	13	14	10	13	14	10
	without	122	154	124	3	6	3	8	8	6	11	12	9	14	14	11

*Averages based on one 3-male mixture set (three donors total)

Table 13. Improved Profile Recovery from Laser Micro-Dissected Buccal Epithelial Cells After Pre-Amplification

Input	Pre-amp?	Avg RFU	Avg # Loci			
			>150	>100	>50	>20
1 cell	with	3054	5	5	5	5
	without	76	0	1	1	1
2 cells	with	4494	9	9	9	9
	without	NA	0	0	0	0
3 cells	with	3537	11	11	11	11
	without	NA	0	0	0	0
5 cells	with	2916	13	13	13	13
	without	110	0	1	1	1
10 cells	with	3659	10	10	10	10
	without	104	1	2	4	5

*Averages based on data from 4 male individuals

Table 14. Improved Profile Recovery from Touch DNA Samples After Pre-amplification

	Pre-Amp																Avg
	DYS456	DYS389I	DYS390	DYS389II	DYS458	DYS19	DYS385		DYS393	DYS391	DYS439	DYS635	DYS392	H4	DYS437	DYS438	DYS448	RFU
door handle	16	13	24	31	17	14	11,14		13	11	12	23	13	12	15	12	19	3742
RFUs	4850	6806	1797	1579	2491	1086	2021	3892	7974	1172	948	7956	4838	2388	6450	4528	2992	
coffee cup	16	13	24	31	17	14	11,14		13	11	12	23	13	12	15	12	19	4173
RFUs	7939	7297	3636	2830	2022	1990	7012	2773	4950	2032	1888	7386	2477	1636	6857	5520	1300	
phone handle	16	13	24	31	17	14	11,14		13	11	12	23	13	12	15	12	19	4261
RFUs	5482	5124	2386	1613	1031	2655	2580	4423	7629	2145	3874	7935	3780	1749	6909	7448	5838	
water jug lid	16	13	24	31	17	14	11,14			11	12	23	13	12	15	12	19	2673
RFUs	3380	3245	4626	1616	339	1237	1244	1520		1839	925	3887	1156	4977	6955	1771	2903	
	No Pre-Amp																	Avg
	DYS456	DYS389I	DYS390	DYS389II	DYS458	DYS19	DYS385		DYS393	DYS391	DYS439	DYS635	DYS392	H4	DYS437	DYS438	DYS448	RFU
door handle																		NA
RFUs																		
coffee cup									13									
RFUs									167									167
phone handle																		NA
RFUs																		
water jug lid																		NA
RFUs																		

Table 15. Profile Recovery from Extended Interval Cervico-Vaginal Samples (6-9 days after intercourse)

The number of alleles recovered from one of the two swabs collected per time interval (6, 7, 8 and 9 days). The shading indicates the average RFU value of all alleles within the profile (white – not detected; light grey < 500 RFUs; dark grey > 1000 RFUs).

Post Coital Interval (Days)	Pre-Amplification?	Allele recovery (out of 17 possible)			
		Couple 1	Couple 2	Couple 3	Couple 4
6	Yes	**17**	**10**	**4**	**17**
	No	**1**	**0**	**0**	**0**
7	Yes	**17**	**16**	**3**	**17**
	No	**0**	**4**	**0**	**0**
8	Yes	**16**	NT	NT	**17**
	No	**0**	NT	NT	**4**
9	Yes	**12**	NT	NT	**14**
	No	**0**	NT	NT	**0**

NT = not tested

Table 16. Improved Profile Recovery from Environmentally Compromised Bloodstains After Pre-amplification

	Pre-Amp																
	DYS458	DYS389I	DYS390	DYS389II	DYS458	DYS19	DYS385		DYS393	DYS391	DYS439	DYS635	DYS392	H4	DYS437	DYS438	DYS448	Avg RFU
M1-37oC-2yr		13	24	31						11	12				15	12	19	6889
		8677	6395	7750						7717	8677				6456	1363	8077	
M1-56oC-2yr					17	14	14		13		12			12	15			7325
					9173	7880		2788	7882		9048			7331	7176			
M1-OSC-6m	16	13	24	31	17	14	11,14		13	11	12	23	13	12	15	12	19	6538
	9021	8740	9069	8739	9460	9491	3887	6232	7920	1242	7062	5351	3131	6945	6914	7879	55	
M1-OSUC-6m										11								2136
										2136								

	No pre-amp																	
	DYS458	DYS389I	DYS390	DYS389II	DYS458	DYS19	DYS385		DYS393	DYS391	DYS439	DYS635	DYS392	H4	DYS437	DYS438	DYS448	Avg RFU
M1-37oC-2yr	16	13							13									71
	87	73							53									
M1-56oC-2yr			24															70
			70															
M1-OSC-6m					17				13	11			13		15	12		205
					307				261	469			63		73	56		
M1-OSUC-6m																		NA

C. FIGURES

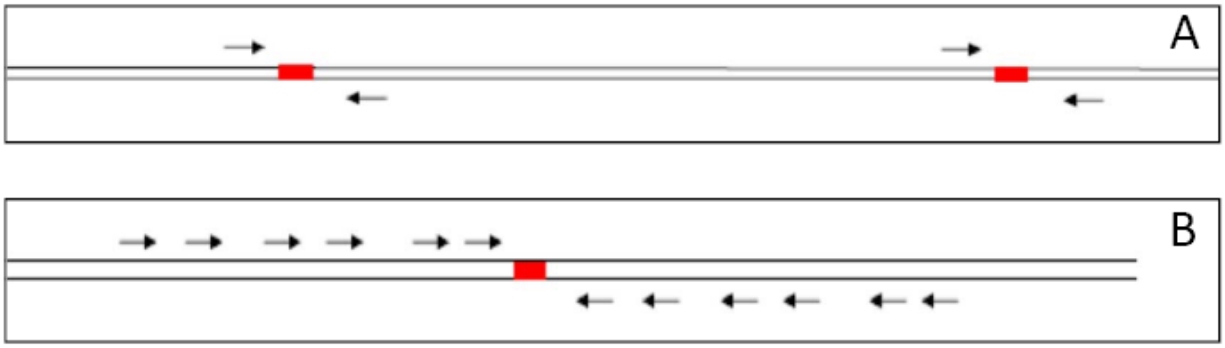


Figure 1. Possible Priming Strategies for use in the Y-chromosome Specific MDA assay. (A) Framing primers, (B) String primers.

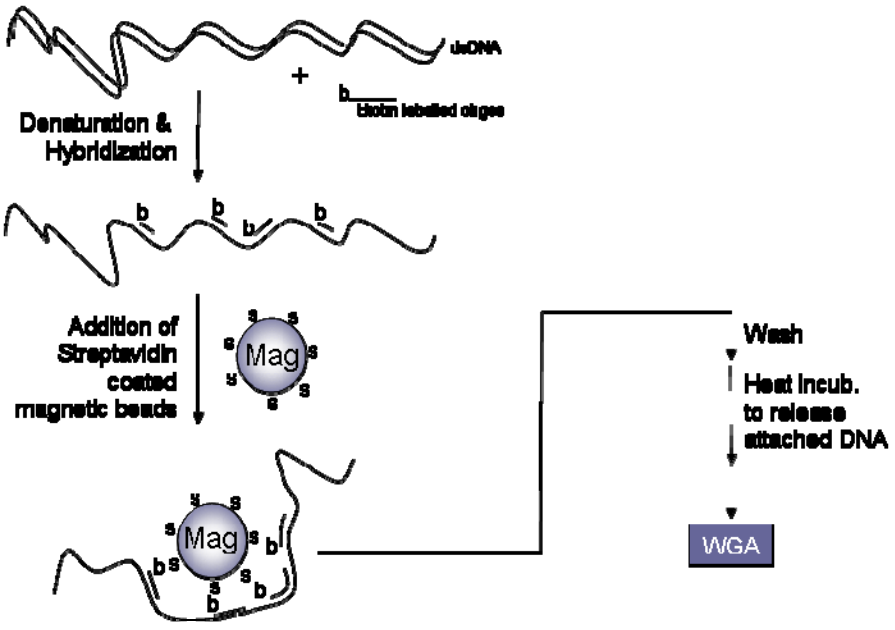


Figure 2. Proposed mechanism to Isolate Y-Chromosomal DNA Using Biotinylated Y-sequence-specific probes.

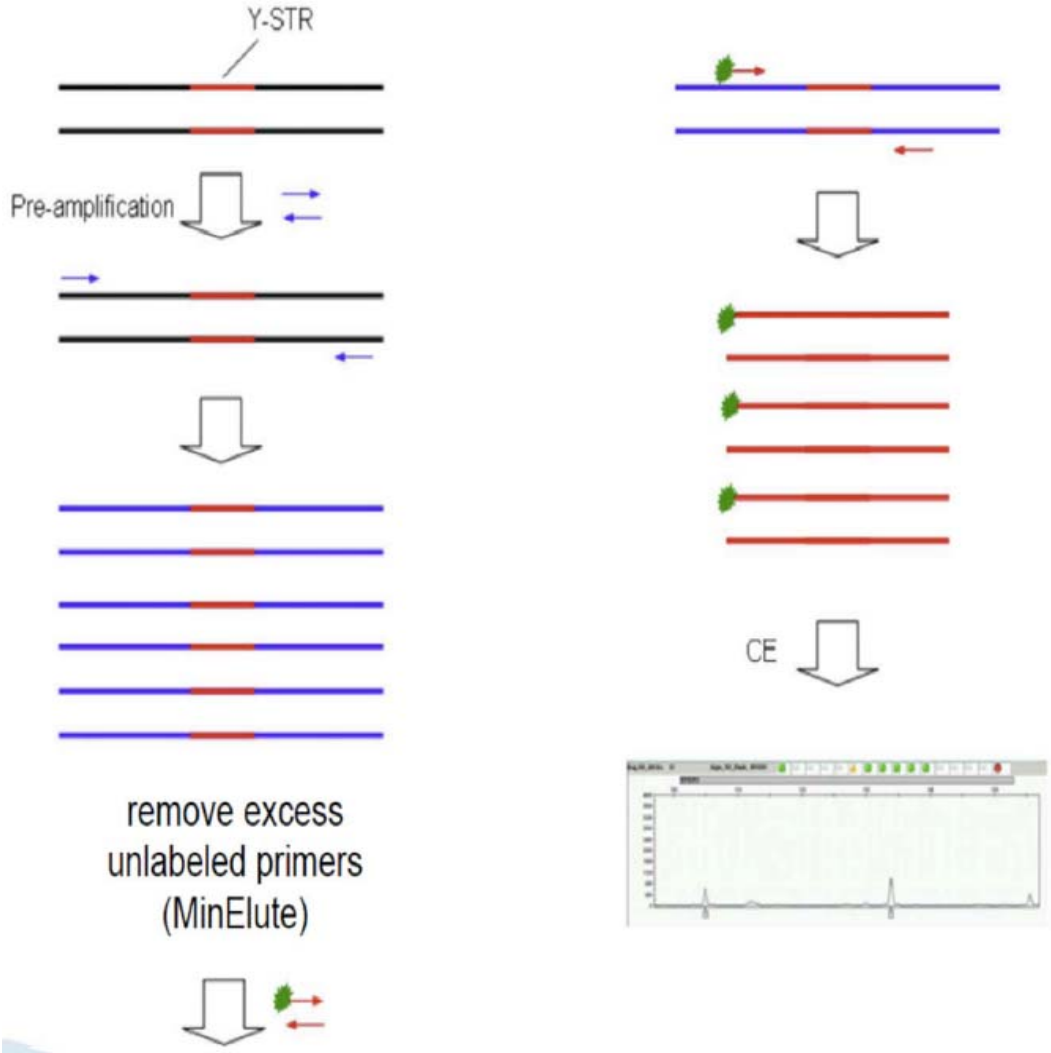
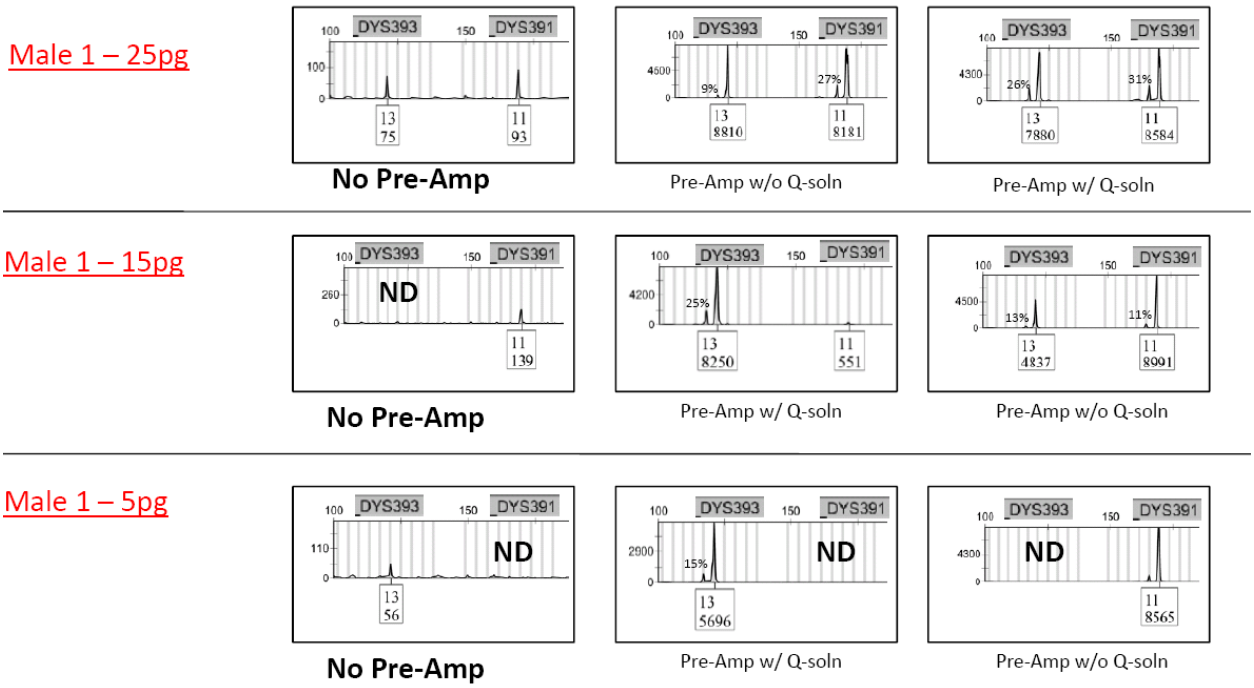


Figure 3. Y-Chromosome Specific Nested PCR Amplification



*ND = not detected

*Stutter percentages shown if detected

Figure 4. Improvement in Allele Recovery with Prior Amplification with the Duplex Nested PCR Pre-Amplification (Yfiler®)

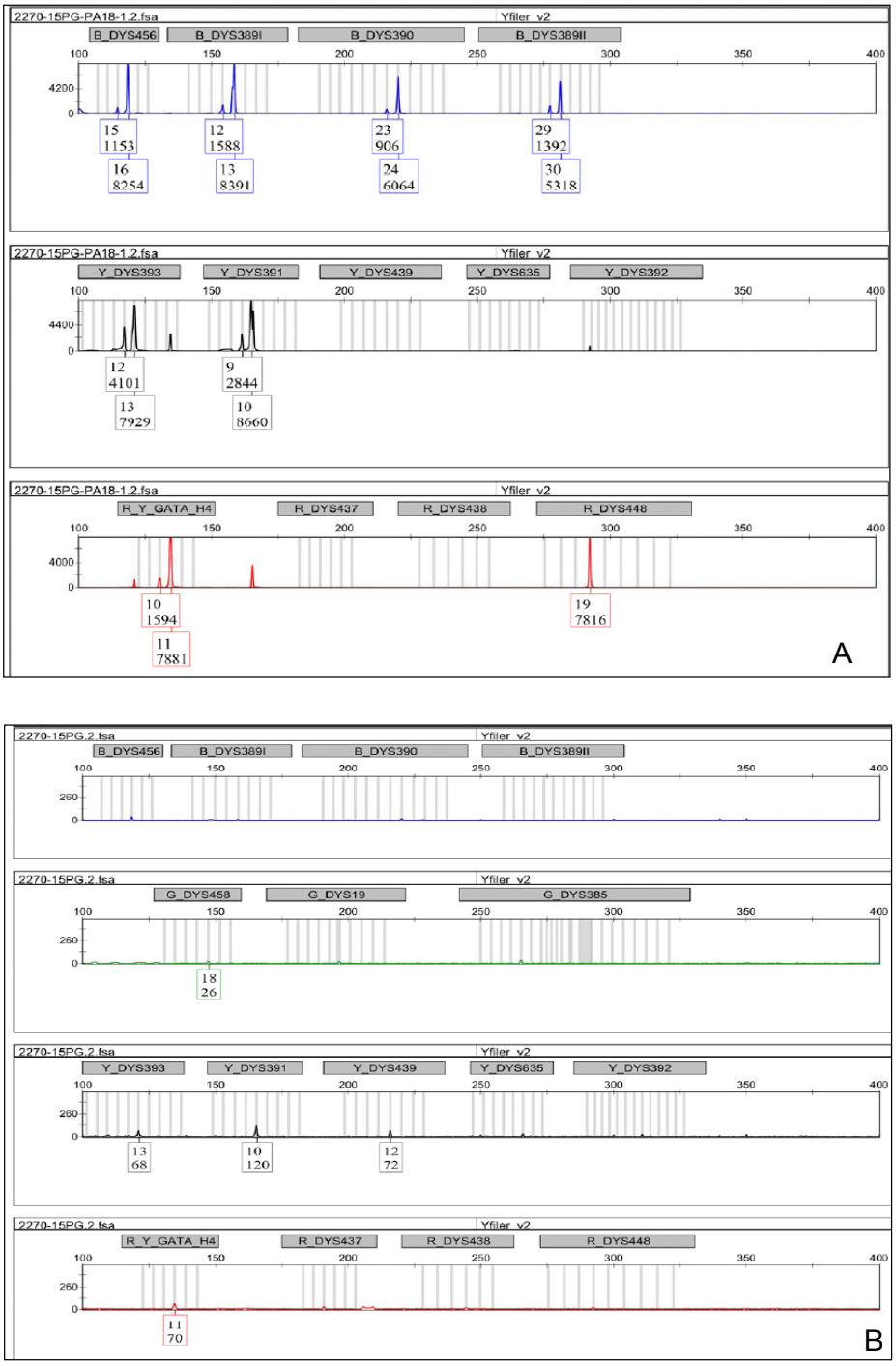


Figure 5. Improvement in Allele Recovery with Prior Amplification with the Advantage HD polymerase 9-plex Pre-Amplification (Yfiler®)

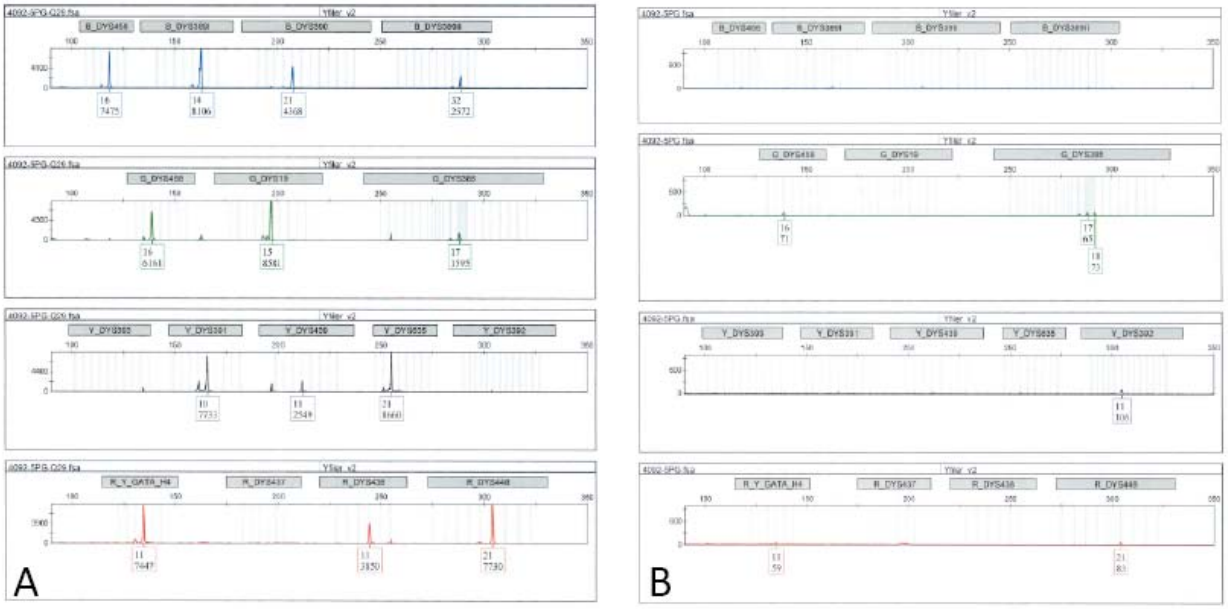


Figure 6. 5pg Male DNA Sample With (A) and Without (B) Prior Amplification With the 17-plex Nested PCR Pre-Amplification Multiplex (Yfiler®)

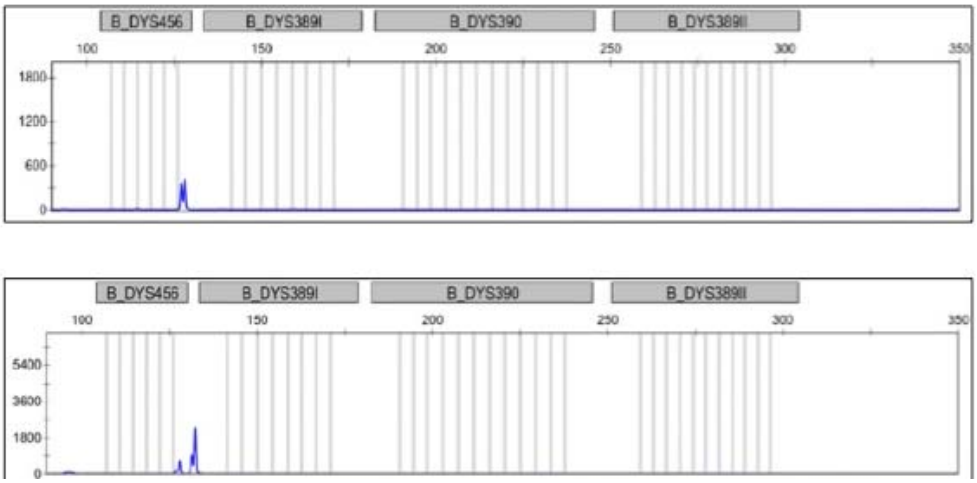


Figure 7. Presence of Female Artifacts after Amplification with the 17-plex Nested PCR Pre-Amplification Multiplex (Yfiler®)

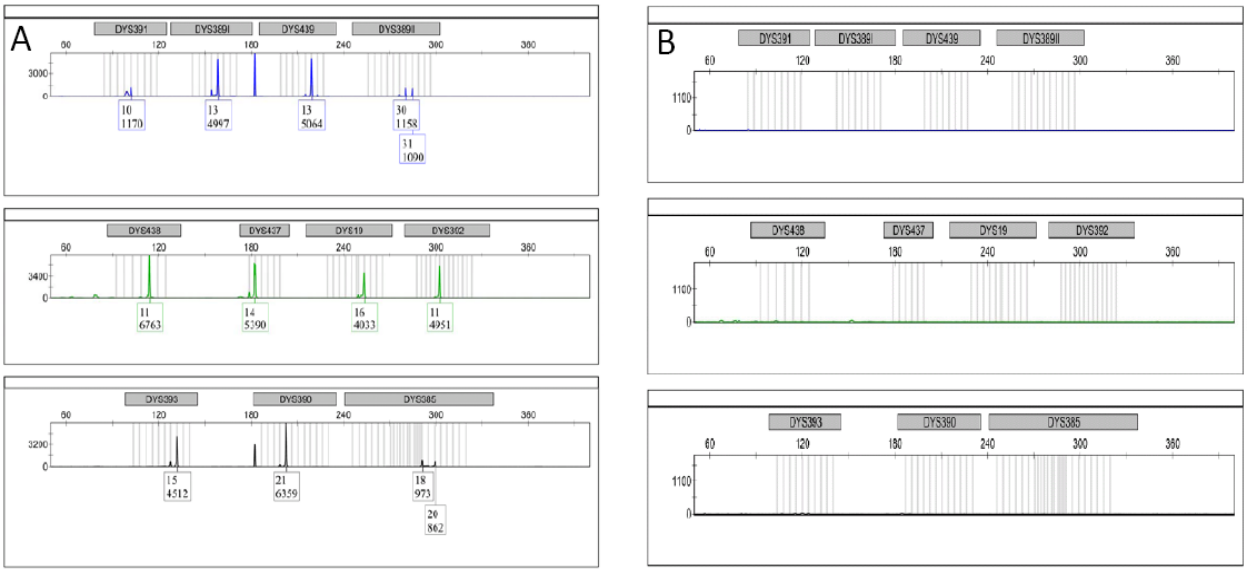


Figure 8. Compatibility of the Nested PCR Pre-Amplification with PowerPlex® Y. A) Profile obtained from 10pg of male input after pre-amplification; b) No detection after Pre-Amplification of ~260ng female DNA

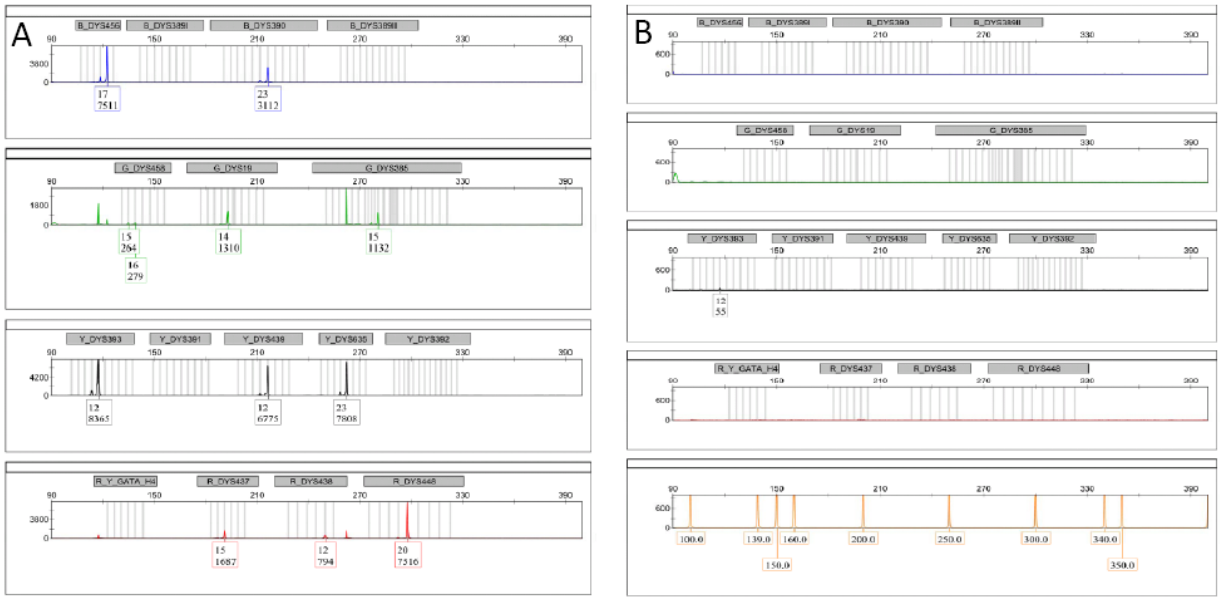


Figure 9. Yfiler® Profiles Recovered from 1 Micro-Dissected Buccal Epithelial Cell With (A) and Without Pre-Amplification (B)

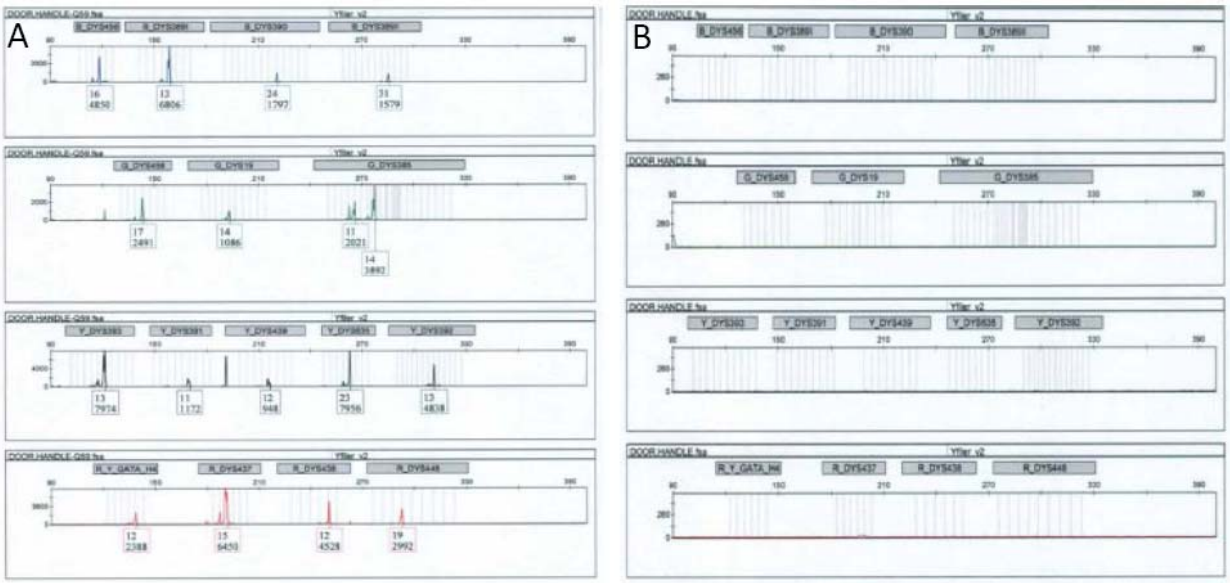


Figure 10. Yfiler® Profiles Recovered from a Swab of a Door Handle With (A) and Without Pre-Amplification (B)

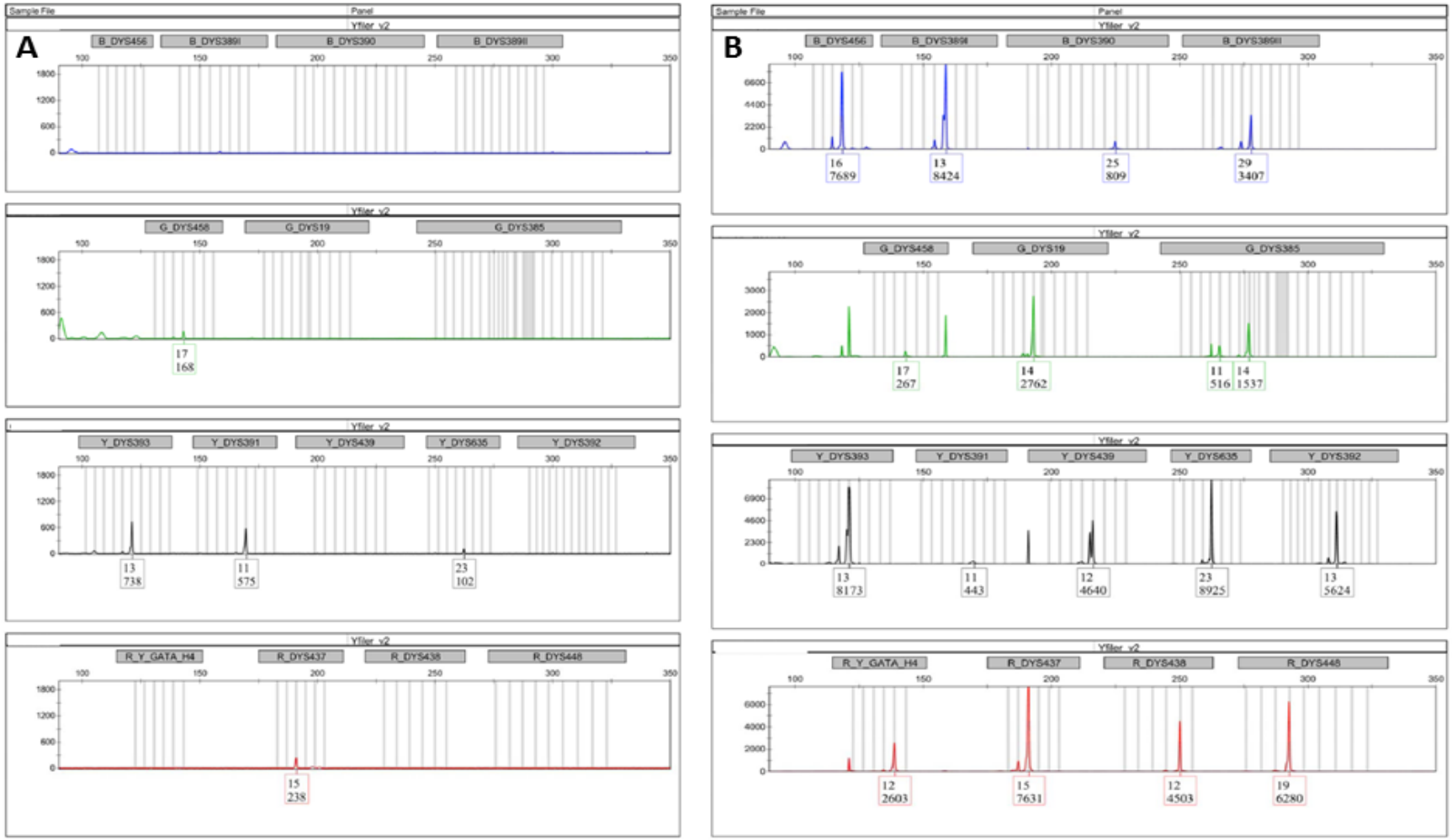


Figure 11. Improved Profile Recovery using Selective Enhancement of Semen Donor – 8 days after intercourse. Y-STR profiles of the semen donor in a 8-day post coital sample without (A) and with (B) pre-amplification. Allele designations are indicated below each locus.

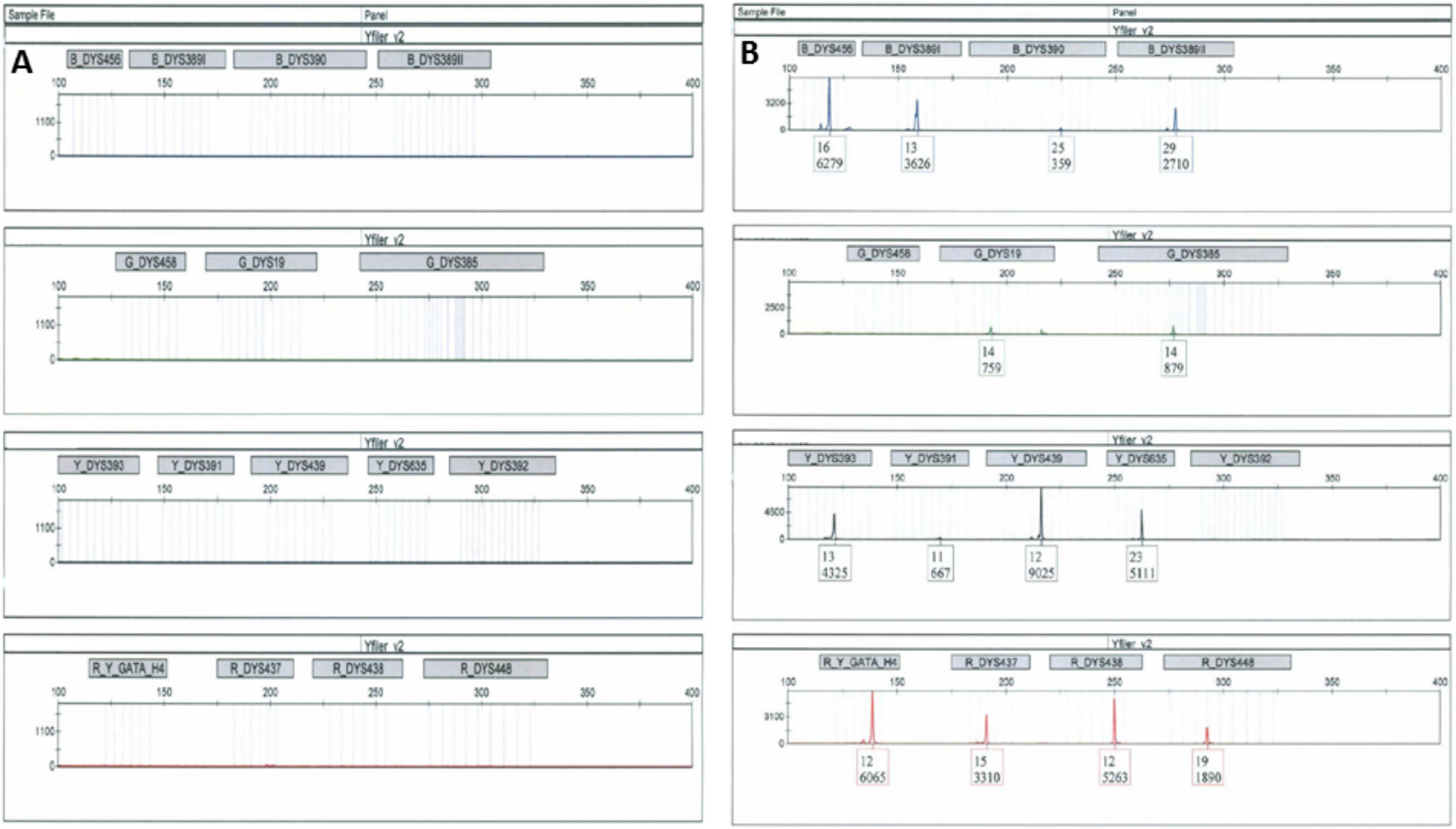


Figure 12. Improved Profile Recovery using Selective Enhancement of Semen Donor – 9 days after intercourse. Y-STR profiles of the semen donor in a 9-day post coital sample without (A) and with (B) pre-amplification. Allele designations are indicated below each locus.

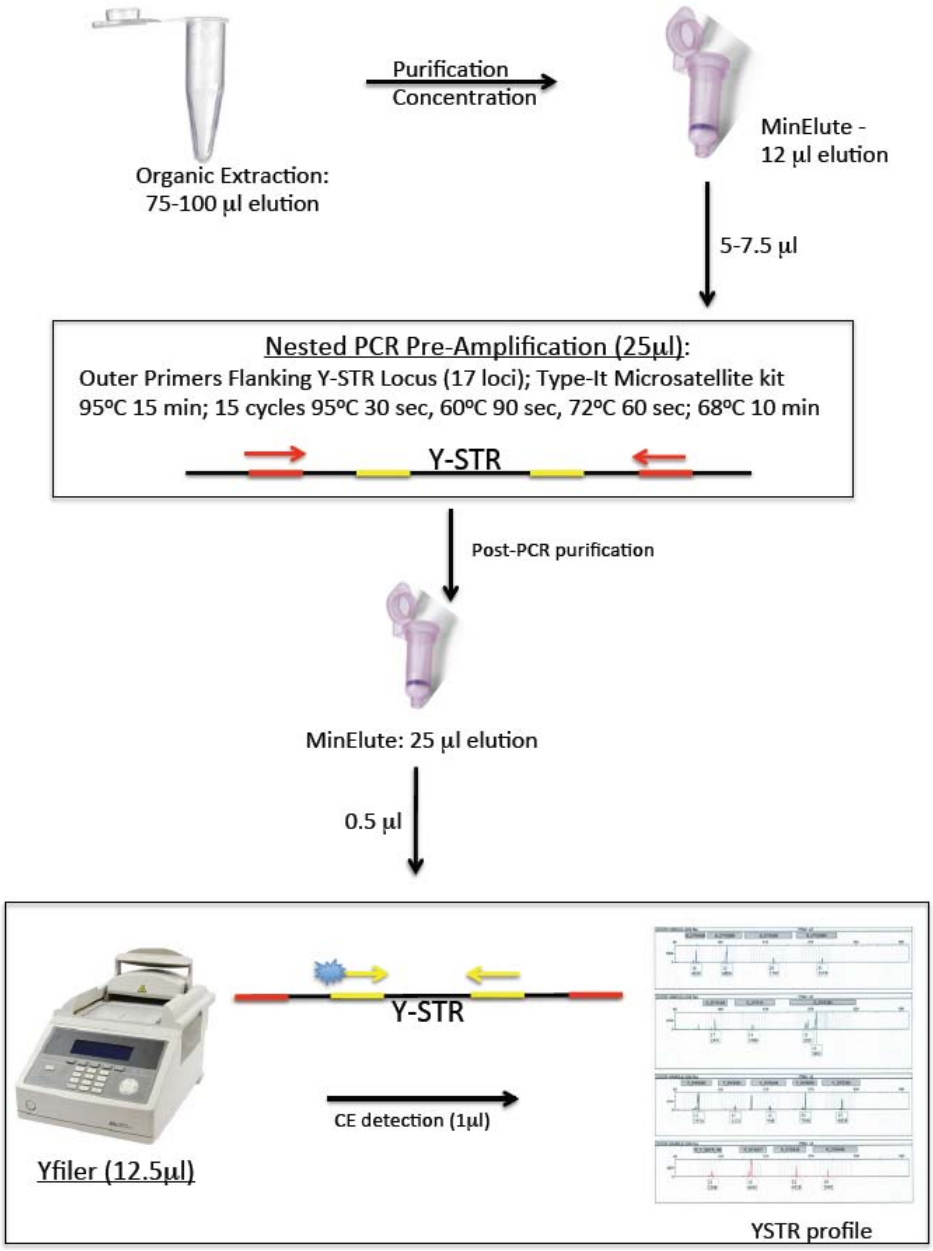


Figure 13. Proposed Schema for the Selective Enhancement of Y-STRs

IV. CONCLUSIONS

A. Discussion of findings

Autosomal STR-DNA profiling has been a standard procedure in forensic laboratories and mitochondrial DNA analysis and Y-STR typing have increased the power of DNA analysis [6-12,37-47]. Methodological advances, such as increased cycle number [13,14], WGA [18-31], and post-PCR purification [15] have improved our ability to recover genetic profiles from evidence. However there are still instances in which a DNA type is not achieved despite the presence of DNA in the samples. This is particularly so when the sample is collected many days after the incident, often as a result of delayed reporting by a rape victim or when there is a significant time interval between death and recovery of a rape/homicide victim's body or when the samples manifest some degree of degradation. In these particular instances, the samples might contain very few male cells admixed with a much larger number of female epithelial cells. Sometimes, a failure to obtain either autosomal or Y-STR profiles from the male donor is observed. While the use of Y-STR analysis should eliminate interference from female DNA, the level of male DNA may fall below the sensitivity limits of the assays used. As a result, methods that permit a selective enrichment of Y-chromosomal DNA might be useful for DNA profiling of these otherwise intractable samples.

The ability to extend sample detection limits for a specific target was previously demonstrated with the REPLI-g® Mitochondrial DNA kit (QIAGEN). This kit uses a multiple displacement WGA method to enrich for human mitochondrial DNA with minimal contamination from nuclear DNA in a simple one-tube procedure. Therefore, the goal of this

project was to utilize the same principle used in the development of this kit for the enrichment of Y chromosomal DNA.

The development of an MDA-based strategy for the enhancement of Y chromosomal DNA proved to be very challenging and, ultimately, was unsuccessful. Our own previous work had demonstrated the ineffectiveness of genome wide MDA with low template DNA [24] and the attempted development of a Y-specific MDA reaction in this work presented additional design challenges particularly in ensuring no primer cross-reactivity with the X chromosome. Despite the lack of success with MDA-based methods, we were able to develop an effective and robust alternative Y-chromosome specific pre-amplification method. The method is based on the use of a multiplex Y-STR-loci-specific nested PCR amplification prior to subsequent standard Y-STR amplification with commercial kits. The nested amplification approach proved to be an effective strategy to increase the signal to noise ratio of Y chromosomal DNA compared with contaminating female epithelial DNA to such an extent as to allow the recovery of Y-STR profiles from otherwise intractable samples. We demonstrate the successful use of this approach with as little as 5 pg of input male DNA. The specificity of the pre-amplification multiplex was demonstrated by the lack of interfering female amplification products. The presence of overwhelming amounts of female DNA in admixed male/female DNA samples did not impede the recovery of male DNA profiles. Probative partial profiles were obtained using as few as 1 buccal epithelial cell after pre-amplification. Full profiles were recovered from contacted or touched objects such as door handles and coffee cups. Despite the larger size of the pre-amplification products, an improvement in allele recovery for samples exposed to 37^{o}C, 56^{o}C and storage outside (exposed to heat, light, humidity and rain) was demonstrated. Remarkably, the use of the pre-amplification multiplex, in combination with extract

purification/concentration, resulted in a significant improvement in the time frame in which male DNA profiles could be recovered from extended interval post coital samples. We demonstrated the ability to obtain full and potentially probative partial Y-STR profiles from samples collected up to 9 days after intercourse. This is a significant improvement in the ability to obtain probative genetic information from late reported sexual assault samples and could have a broader impact on the time frame during which sexual assault evidence should be collected.

Summary of the Method

A summary of the method for selective enhancement of Y chromosomal DNA is provided in Figure 13. The method is relatively easy to perform and would require little to no additional training or equipment for operational crime laboratories. As can be seen from Figure 13, biological samples are extracted with a standard non-differential organic extraction, even for semen containing evidence thus eliminating the need for differential extractions in which male DNA can potentially be lost during the physical manipulations of the extraction. For challenging samples, such as extended interval post coital samples or touch DNA samples, an entire swab should be used for extraction to ensure that the maximum amount of male DNA is recovered. A standard elution volume of 75 – 100 µl TE-4 is used in order to ensure efficient and complete DNA re-solublization, particularly in the cases of admixed samples in which an overwhelming amount of female DNA is present. The entire sample extract is then purified and concentrated using the MinElute purification system (QIAGEN). The use of a 12 µl elution permits a portion of the extract to be available for quantitation (2µl) and pre-amplification (5-7.5 µl, typically 5 µl) which typically leaves ~ 5 µl as a duplicate sample for subsequent re-analysis.

It was our experience that much of the male DNA tested was present in such low quantities even after concentration that it was undetectable using commercially available real time PCR quantitation assays. Therefore, if a quantitation is performed as required by National standards, an undetectable result should not preclude the samples from being pre-amplified. The pre-amplification multiplex is a 25 µl amplification reaction that utilizes that Type-It Microsatellite mix (commercially available from QIAGEN) and 15 primer sets that permit the pre-amplification of the 17 commonly used Y-STR loci (the two bi-local loci, DYS389 and DYS385, are each amplified by a single primer set). The pre-amplification primers (shown in red in Figure 13) are located in regions flanking the Y-STR locus of interest (shown in yellow in Figure 13). They are located far enough outside of the Y-STR locus to accommodate the use of various commercial Y-STR kits, which contain primers with different sequences. The pre-amplification is a short 15-cycle amplification program that can be completed in less than two hours. Following pre-amplification, the products are post-PCR purified with the MinElute purification system using a 25 µl elution (i.e. sample clean-up with no sample concentration). A small aliquot of the purified pre-amplification product (0.5/25 µl, or 2%) is used in a subsequent reduced volume Yfiler amplification (12.5 µl from the standard 25 µl reaction) using fluorescently labeled Y-STR specific primers from a commercial kit (shown in yellow in Figure 13). The products are then detected using capillary electrophoresis. A significant portion of the pre-amplification product is available for re-testing, although this is limited to Y-STR analysis since the sample has been pre-amplified. Any portion of the original purified and concentrated extract not added to the pre-amplification multiplex would be available for other non-YSTR testing or for a second pre-amplification reaction if needed. This pre-amplification portion of the process can be completed in a single day and portions of it can be automated or at least semi-

automated, such as the MinElute purifications (performed on the QIACube in this work). Currently, the pre-amplification primer mix is prepared in-house. However, there is potential for the pre-amplification multiplex to be made commercially available, thereby facilitating the implementation of this method into forensic casework.

Despite the success of the Y-chromosome specific nested PCR pre-amplification method demonstrated here, there is likely to be initial concerns regarding an increased risk of contamination due to an increase in cycle number and need for sample manipulation during purification and secondary amplification steps. While there is always a potential risk for additional contamination when a larger number of amplifications cycles are used, significant contamination issues were not observed throughout the course of this study. As mentioned previously, extraction blanks were subjected to the same analysis as the samples. This included extract concentration, pre-amplification, purification and subsequent Y-STR amplification and detection. Contamination was not observed in any of the extraction blanks and drop-in alleles (not originating from the sample donor) were rarely observed. Therefore, it should be possible, with proper controls and procedures to employ these methods without significant contamination issues. Once the pre-amplification reaction has been performed, further processing of the samples (purification and Y-STR amplification) would have to occur in a post-amplification room. It is possible that small separate bench-top PCR workstations could be designated for purification set-up as well as secondary amplifications in order to isolate these reactions from other areas of the post-amplification environment and minimize contamination. Other standard practices of minimizing the time and frequency of tubes containing amplified product, sterilization of pipets and work spaces, and use of sterile consumables and reagents should also reduce the risk of potential contamination. As further validation work is performed on this

method, the potential risk for contamination and additional strategies or method modifications to try to reduce that risk will be explored.

While there is a potential risk for contamination due to the increased number of amplification cycles used with a nested PCR approach as well as additional sample manipulation required for the purification and secondary amplifications, as described above, with proper procedures and controls the risk can be reduced. However, there is an additional challenge with the nested PCR approach since STRs are the regions of interest. Similar to what is observed with standard STR analysis, the first round amplification would still be subjected to amplification artifacts such as stutter (n-1 and n+1). While the use of the second round amplification reduces or eliminates amplification of non-specific amplification products that do not contain the "nested" primer sequences, stutter products would contain these primer binding sites and therefore would be amplified as well. Therefore, there is an increased risk of stutter products being amplified which could result in high RFU products which would be well above normal stutter percentages for an STR locus. A possible example of this can be seen in Figure 8 (DYS389I locus, PowerPlex® Y) where both 30 and 31 alleles were present. The 31 allele is the correct type for the donor used in this study. While the 30 allele may appear to be contamination or drop-in, it is possible that this is a stutter product that has been "enhanced" as well as the true allele. While potential increased stutter peaks were not frequently observed, a detailed stutter study was not performed in this initial development work but is critical to include in a future full developmental validation. The benefit of having a separate first-round amplification (as opposed to simply using increased cycle number with the same kit or using a kit as the pre-amplification) is that the reaction components and additives can be modified to improve efficiency and reduce artifacts. For example, enzymes with high fidelity and processivity can be evaluated in the pre-

amplification multiplex that would ideally provide reduced change of stutter products being formed. This would have to be empirically tested of course and the authors fully acknowledge that such an approach may not provide a reduction in stutter products as this is a significant challenge in forensic DNA analysis and understandably not one easily solved. However, there is a need to try and reduce stutter in standard DNA analysis as well as with LTDNA analysis.

B. Implications for policy and practice

The ability to provide investigators with a DNA profile from a male donor of a deposited biological stain has become an essential part of the investigation and prosecution of crimes. There remain, though, some cases where the ability to obtain an unambiguous male DNA profile is still problematic due to sample quantity or integrity problems. Pre-amplification of Y specific chromosomal DNA in challenging evidence samples containing male/female DNA mixtures, as demonstrated here, permits the ability to obtain a male donor DNA profile from otherwise intractable samples. The success of this work heralds a possible new tool that could be made available to the forensic biologist to aid them in recovering potentially probative DNA profiles from a sub-set of otherwise intractable samples.

C. Implications for further research

The Y-chromosome specific nested PCR pre-amplification multiplex permits Y-STR profiles to be obtained from extended interval post coital samples and touch DNA samples. In many instances no profiles would have been recovered from these samples without prior pre-amplification. Additional validation work is needed in order to evaluate *bona fide* casework samples. Additionally only a small number of touch DNA samples were evaluated. Future work

should be performed to determine if the nested PCR pre-amplification multiplex could be used routinely for the analysis of touch DNA samples. It may also be possible to apply the strategies developed in this study to autosomal STR typing to further improve the analysis of touch DNA samples.

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VI. DISSEMINATION OF RESEARCH FINDINGS

A. Publications

1. "Y-Chromosome Specific Nested PCR Pre-Amplification Method for Improved Detection of Male DNA." Hanson, E., DiPasquale, F., Strauss, S., Engel, H., and Ballantyne, J.
   *This manuscript is in preparation and is expected to be submitted for publication to Forensic Science International Genetics in April 2012.
2. "Analysis of Late Reported (>6 days) Sexual Assaults by Y-chromosome Specific DNA Enhancement". Hanson, E and Ballantyne, J.
   *The manuscript has been prepared and is currently being edited before submission to Medicine, Science and the Law. It describes a specific application of the Y-chromosome specific nested PCR pre-amplification to extended interval post coital samples. Expected submission date: February 2012.
3. Assessment of DNA degradation and the genotyping success of highly degraded samples. Hughes-Stamm, S., Ashton, K., and van Daal, A., Int J Legal Med 125(3), p 341-348. (2011)

B. Presentations

1. Improved Detection of Male DNA from Post-Coital Samples. Ballantyne, J. and Hanson, E. The NIJ Conference, Crystal City, VA. (2008)
2. Measures of DNA degradation and the Predictive Genotyping Success of Highly Degraded Samples. Hughes-Stamm, S., Ashton, K., van Daal, A. Australian and New Zealand Forensic Science Society, Melbourne, Australia. (2008)
3. Improved Detection of Male DNA From Post Coital Samples. Hanson, E., Korfhage, C., Loeffert, D., and Ballantyne, J. The Annual NIJ Conference, Washington, D.C. (2009)
4. Assessment of DNA degradation and the genotyping success of highly degraded samples. Hughes-Stamm, S., Ashton, K., van Daal, A. International Society for Applied Biological Sciences conference, Split, Croatia. (2009)
5. Forensic Biology Research at the National Center for Forensic Sceince. EDNAP (European DNA Profiling Group) Meeting, Kieve, Ukraine. (2010)
6. Forensic Biology Research at the National Center for Forensic Science. EDNAP (European DNA Profiling Group) Meeting, Brussels, Belgium .(2011)
7. The effects of Y-STR Research on Practice and Policy. Ballantyne, J. The Annual NIJ Conference, Washington, D.C. (2011)
8. Enhanced DNA Profiling of the Semen Donor Can Assist the Investigation of Late Reported (>5 days) Sexual Assaults. Hanson, E. and Ballantyne, J. American Society of Crime Lab Directors (ASCLD) Annual Symposium, Denver, CO. (2011)
9. Y-Chromosome Specific Nested PCR Pre-Amplification Method for Improved Detection of Male DNA. Hanson, E., Solivan, M., Strauss, S., Dr Pasquale, F., Engel, H., and Ballantyne, J. 22nd Annual Symposium on Human Identification, National Harbor, MD. (2011)
10. Y-Chromosome Biology and the National Y-STR Database. Ballantyne, J. Y-Chromosome Short Tandem Repeat (Y-STR) Analysis and Typing, California Criminalistics Institute, Richmond, CA (webinar). (2011)

11. Enhanced DNA Profiling for Detection of the Male Donor in Trace Samples. Hanson, E. and Ballantyne, J. European National Forensic Science Institutes (ENSFI) DNA Working Group Meeting, Athens, Greece. (2011)

Appendix A: Bond University Final Report

FINAL REPORT

Date: 10 December 2010

SECTION A - Administration

Type of Research Grant	National Institute of Justice
Award Number	2007-DN-BX-K147
Research Grant Title	Y Chromosome Whole Genome Analysis Strategies: Improved Detection of male DNA
Chief Investigator Bond University	Prof Angela van Daal
Research staff	Most of this work was conducted by a Graduate Student (Sheree Hughes-Stamm) as part of her PhD thesis. Casual Research Assistants were employed July – August 2010
Administering Institution	University of Central Florida
Year Funding Commenced	2008

SECTION B – Results

1. GENERATION OF DEGRADED DNA SIZE CLASSES

In order to investigate the potential for amplification of low levels of Y chromosomal material that may additionally be degraded it was necessary to generate a series of DNA fragments of different size classes. The size classes initially generated were:

- Greater than 400bp
- Between 200bp and 400bp
- Less than 200bp

DNA was extracted from whole blood using QIAamp® Blood Maxi Kit (Qiagen, Hilden, Germany). Three methods were used for generating these DNA fragment size ranges. These were:

a) Physical degradation by sonication
b) Enzymatic degradation with DNAse I
c) A combination of physical and enzymatic degradation using sonication and DNAse I

1.1 METHOD OF DNA FRAGMENTATION

1.1 a) Sonication

Sonication was conducted using a Branson SLPt sonifier and the following parameters were assessed with 0.5 – 1.5mL high concentration DNA sample

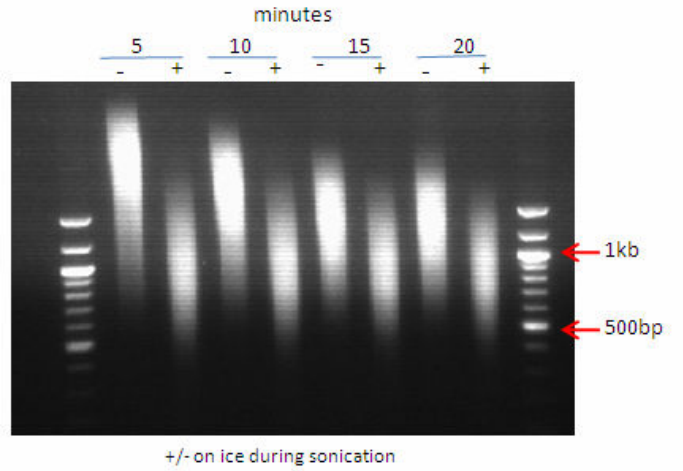
- Sample Volume (500 – 1500uL)
- Amplitude (20-90%)
- Time (0 – 60mins)
- Continuous sonication vs Pulse (on and off intervals)
- On ice vs not on ice
- Frothing vs no production of froth

Volumes above 1000uL were found to spill, froth more and take longer to fragment. Sonicating samples for longer than 25mins was found to result in no additional DNA fragments fragmentation. Additionally conditions that induced heat or frothing in the DNA sonication process reduced the efficiency of fragmentation. These included continuous sonication (without any off period), high amplitude (<60%) and not being on ice during the sonication treatment.

The level of DNA degradation (fragment size range) was assessed by 1.5% agarose gel electrophoresis or by visualisation on the QIAxcel capillary electrophoresis system (QIAGEN).

Figure 1.1A. DNA Sonication Time Series: Agarose Gel

1 mL of high concentration DNA was sonicated for 5, 10, 15 and 20 minutes on ice (+) or not on ice (-). Aliquots of each were analysed on a 1.5% agarose gel.



DNA samples were also analysed via capillary electrophoresis using a High Resolution gel cartridge on a QIAxcel system (Qiagen, Hilden, Germany). Aliquots (1-3µL) of DNA extract were combined with DNA dilution buffer (Qiagen) to 10µL total volume. The QIAxcel system produces a digital gel image and an electropherogram for fragment analysis.

The optimal protocol for generation of DNA fragmentation by physical means was:

- Sample Volume of 1mL (in a 2ml Eppendorf tube)
- Amplitude 50%
- Sonication Time 0-25mins
- Pulse sonication: 30sec on/30 off cycles
- On ice with probe just under the meniscus

Figure 2 shows the three levels of DNA degradation that were generated:

- Moderate – 5 minutes sonication
- High – 10 – 15 minutes sonication
- Extreme – 20 – 25 minutes sonication

Figure 1.2A. Levels of DNA Degradation

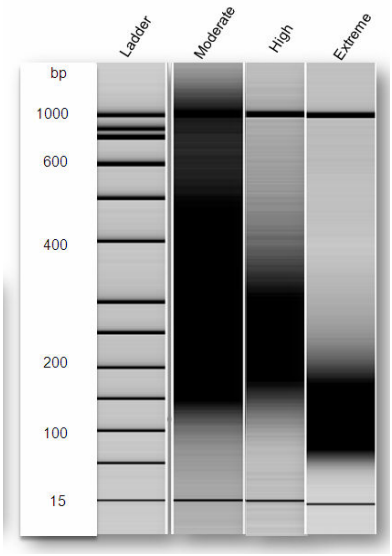
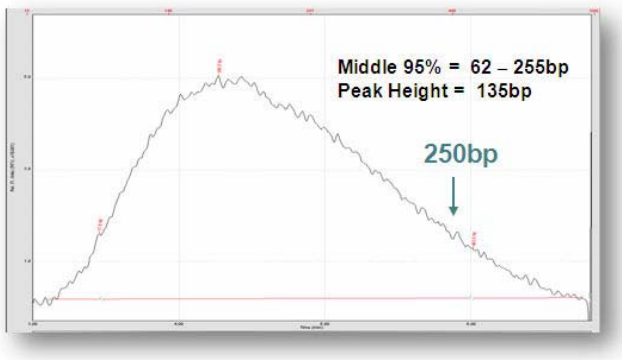


Figure 1.2 B. High Level of Degradation

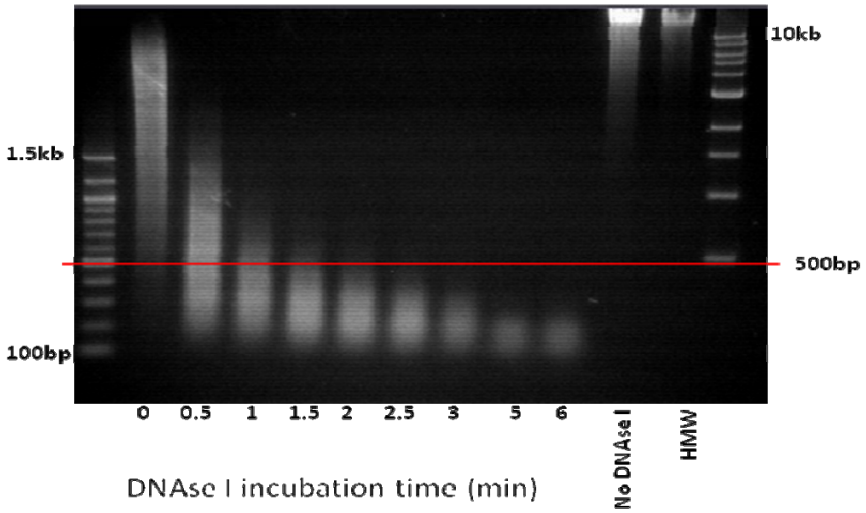


1.1 b) DNAse I

Initial experiments using DNAse I resulted in either no degradation or complete degradation (results not shown). Subsequent work with DNAse I resulted in good ranges of DNA fragmentation.

The optimal protocol for generation of DNA fragmentation by enzymatic means was:
1U DNAse I (Promega) added to 500uL reaction volume (50uL DNase I Reaction Buffer + 450uL neat DNA extract) incubated at 37°C with 50uL aliquots taken at each time period. The DNAse reaction was stopped with the addition of 1uL 20mM EGTA and inactivated at 65°C for 10minutes.
In order to generate sufficient quantities of DNA working stocks on which to test different repair conditions several of these DNAse reactions would be performed per DNA sample and then pooled into appropriate sized sample ranges.

Figure 1.3. DNAse Degradation Time Series: Agarose Gel

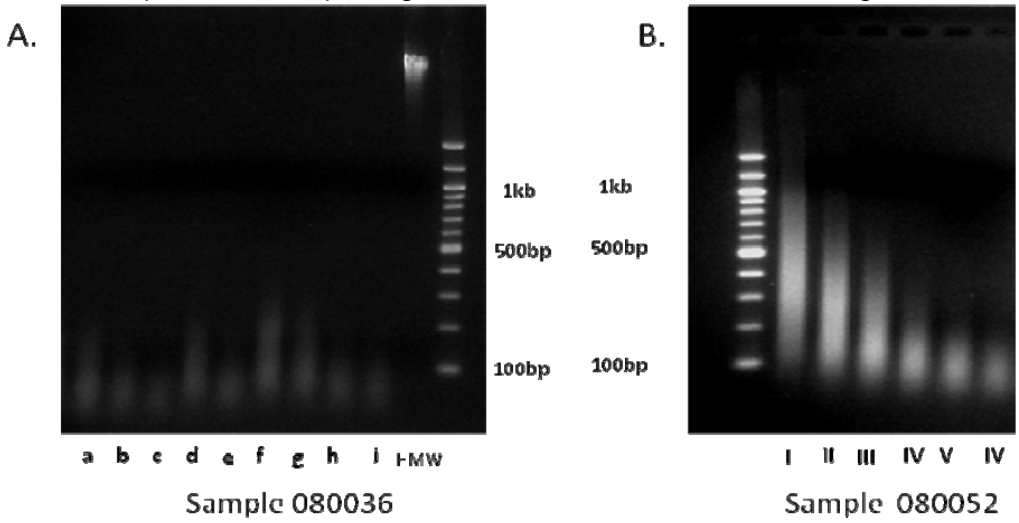


Several of these runs would be performed per DNA sample and then pooled into appropriate sized samples to generate working stocks on which to do repair.

Six levels of degradation, I – VI, were generated using DNAse I.

Figure 1.4. Pooled Degradation Samples

A. Example of one sample degraded in several reactions that were subsequently pooled to create a stock of DNA degraded to a particular level.
B. Example of one sample degraded to the six different levels of degradation.



1.1 c) DNAse I + Sonication

Samples were degraded using both DNAse I and sonication. There was no improvement seen with the DNA degradation than with sonication or DNAse I separately (data not shown).

1.2 THE DEGRADED DNA SAMPLES

A series of samples of varying levels of degradation have been generated. Samples were generated with sonication or DNAse. A series of artificially degraded samples ranging from moderate to extreme levels of degradation was achieved with 5 min, 15 min and 25 min sonication respectively.

Table 1 illustrates the samples generated by sonication.

Table 1 Code:
Each sample had a 3 digit code.

- The first letter represents the sample ID.
- The second letter represents the level of degradation (M = moderate, H = high, E = extreme).
- The third digit represents the extraction batch (1 = first batch, 2 = second batch etc)

DNA SAMPLE DEGRADATION SERIES BY SONICATION					
SEX	**SAMPLE ID**	**MODERATE** **>400bp**	**HIGH** **200-400bp**	**EXTREME** **<200bp**	**UNDEGRADED** **HIGH MOLECULAR WEIGHT**
		12 Samples: 4 ♀ 8 ♂	9 Samples: 2 ♀ 7 ♂	12 Samples: 2 ♀ 10 ♂	16 Samples: 5 ♀ 11 ♂
Male	N	NM1; NM2	NH1	NE1 NE2	NHMW1; NHMW2
	Degradation Ratio			3.4	
	K	KM1	KH1; KH2	KE1	KHMW1; KHMW2
	Degradation Ratio			3.62	
	P	PM1	PH1	PE1 PE2 PE3	PHMW1; PHMW2
	Degradation Ratio			3.71	
	E	EM1	EH1	EE EE2	EHMW1
	Degradation Ratio			3.47	
	S	SM1	SH1	SE1	SHMW1; SHMW2
	Degradation Ratio			2.81	
	AN	ANM1; ANM2	ANH1	ANE1	ANHMW1; ANHMW2

Female	KE (♀)			KEE1	KEHMW1
	Degradation Ratio			3.81	
	R (♀)	RM1	RH1; RH2	RE1	RHMW1
	Degradation Ratio			2.03	
	KA (♀)	KAM1			KAHMW1; KAHMW2
	AL (♀)	ALM1; ALM2			ALHMW1

2. ASSESSMENT OF DNA QUALITY

Degradation was assessed in a two-step process. The first step involved fragment analysis using the QIAxcel capillary electrophoresis system. The second step involved quantitative and qualitative analysis by a qPCR assay.

2.1 QIAxcel Fragment Analysis

The QIAxcel system (Qiagen) produces a digital gel image for fragment analysis (Figure 1.2A). The electropherogram provides a profile of the fragment composition of each sample (Figure 2B). Samples with greater than 90% of fragments sized below 200bp were considered extremely degraded. Those samples with more than 90% of fragments sized between 200bp and 400bp were considered highly degraded and samples with greater than 90% of fragments sized above 400bp were considered moderately degraded. The STR (100-400bp) genotyping method was used to assay genotyping success of the repair treatments.

2.2 qPCR Method

The qPCR assay that was developed is described in **Hughes-Stamm**, SR Ashton, K and van Daal, A (2010) Int J Leg Med. Apr 24. [Epub ahead of print] and text from this publication is reproduced. The four targets of the qPCR assay included two autosomal (TH01 and CSF), one male-specific target (SRY) and a synthetic oligonucleotide internal PCR control (IPC). PCR product was detected using dual labelled hydrolysis probes (TaqManMGB®, Applied Biosystems, Foster City, CA and TaqMan®, Operon, Huntsville, AL). The assay was performed on the Rotor-Gene 6000 (Qiagen) real-time thermocycler in a 20µL reaction volume using QuantiTect Multiplex PCR Mastermix (Qiagen). Modifications to the previously published assay include 5'dyes and primer concentrations (Hudlow WR, Chong MD, Swango KL, Timken MD, Buoncristiani MR (2008) *Forensic Science International: Genetics* 2(2):108-125). Primer and probe sequences were unchanged as in Hudlow *et al.* except for the SRY probe ([Cy3.5]TTGCCCTGCTGATCTGCCTCCC[BHQ2A]). The two-step qPCR protocol consisted of an initial 15min 95°C polymerase activation step, followed by 40 cycles of 60 sec of denaturation (94 °C) and 90 sec of combined annealing/extension (60°C). Pre-quantified, high molecular weight human genomic male DNA (Promega, Madison, WI) was used as a qPCR quantification standard and no template controls were included to monitor contamination. The ability of the assay to quantify the amount of male DNA in a background of female DNA was assessed by combining male and female DNA (1:1, 1:10, 1:100 and 1:1000). Both undegraded high molecular weight (HMW) and degraded mixture samples were tested. HMW mixtures utilised Human Genomic Male DNA (Promega, Madison, WI), and female K562 DNA (Promega, Madison, WI). Degraded male and female DNA as described above were used to assess the qPCR assay.

2.3 qPCR Degradation Assay Results

The modified quadruplex qPCR assay was able to simultaneously quantify total human DNA, male DNA and the extent of PCR inhibition and DNA degradation with reasonable accuracy and sensitivity. Calibration curves for each assay, namely; TH01, SRY, CSF, IPC showed good linearity with R^2 values above 0.99. Between assay reproducibility was assessed with three separate assays each in triplicate and data for each sample were used to calculate the mean and standard deviation.

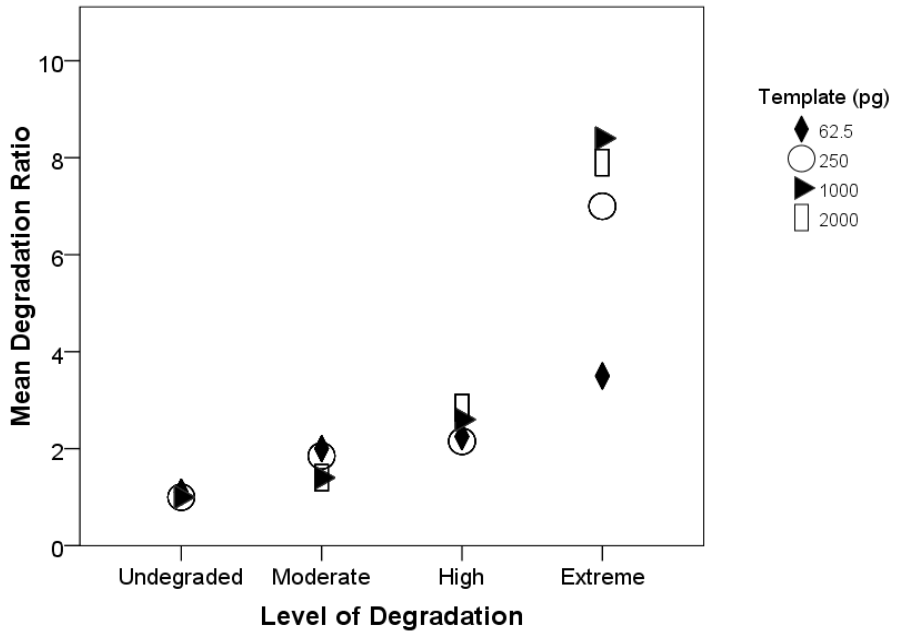
The real-time quantitative PCR (qPCR) assay that was developed (**Hughes-Stamm**, SR Ashton, K and van Daal, A (2010) Int J Leg Med. Apr 24. [Epub ahead of print]) was used to simultaneously quantify the DNA, assess the degradation level of the DNA and measure the level of PCR inhibition of the DNA sample. The total amounts of human nuclear DNA and Y chromosomal DNA were assessed using respectively primers to autosomal STR regions (TH01 and CSF) as well as to a region specific for the Y chromosome (SRY). PCR inhibition

was monitored using a synthetic oligonucleotide internal PCR control (IPC). The level of DNA degradation was measured by the ratio of the TH01 and CSF amplicons. The larger TH01 amplicon (170-190bp) is seen in decreasing levels relative to the smaller CSF amplicon (67bp) with increasing levels of DNA degradation. The SRY amplicon is 137bp and also can be used to measure the level of degradation in male samples.

The extent of DNA degradation could be assessed with reasonable accuracy to 62.5pg and genomic targets could be quantified to a lower limit of 15.6pg. The qPCR assay was able to detect male DNA to a lower limit of 20pg in a 1:1000 background of female DNA.

The ability of the CSF-TH01-SRY-IPC assay to indicate the level of DNA degradation in a sample showed that, as expected, the degradation ratio (CSF quantity/ TH01 quantity) increased as the level of degradation increased (Figure 2 reproduced from Hughes-Stamm et al). The CSF-TH01-SRY-IPC assay is able to predict the degree of degradation with DNA template amounts from 2ng – 15.6pg in high quality samples and to a level of 62.5pg for degraded samples.

Figure 2.1. qPCR Assay Sensitivity for DNA Degradation Assessment
A ratio of 1 in the qPCR assay indicates equal amplification of both long and short PCR targets. A ratio >1 indicates greater amplification of the smaller target and therefore possible DNA degradation.



3. WHOLE GENOME AMPLIFICATION

3.1 Methods

3.1.1 Whole genome amplification (WGA)

Whole genome amplification was performed using the Genomiphi V2 DNA Amplification Kit (GE Healthcare,Buckinghamshire,UK). The WGA uses the multiple displacement amplification (MDA) method. 5µL (10ng and 1ng) template (degraded DNA, Restorase, PreCR, T4 or CircLigase treated samples) was added to 5µL sample buffer. This mix was denatured at 95% for three minutes and cooled on ice. After addition of 9µL of reaction

buffer and 1µL of enzyme mix, samples were incubated at 30° for 90 minutes and the enzyme was then inactivated by incubation at 65°C for ten minutes.

- *Variables investigated:*
  Input DNA volume (1, 3 or 5µL DNA to total reaction volume of 10uL)
  Inclusion of molecular crowder (PEG) 1-5%
  With/without EtOH precipitation prior to PCR

  +/- filtrations and concentration prior to WGA

3.1.2 Formalin-fixed , paraffin embedded (FFPE) Repli-g

The commercial FFPE-Repli-g kit (QIAGEN) allows whole genome amplification (WGA) of highly fragmented DNA isolated from FFPE samples. Reactions using artificially degraded DNA (100ng) were performed following the manufacturer's recommended instructions for amplification of purified genomic DNA. Samples were incubated for the standard time of two hours at 30°C. 1µL of FFPE product was added to downstream assays.

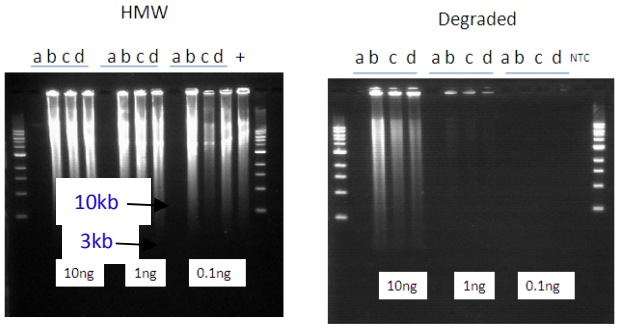
- *Variables investigated: High yield eight hour incubation at* 30°C

3.2 Results

3.2.1 Whole genome amplification

To allow addition of maximal repair product to the WGA reaction, the tolerance of the GenomiPhi kit for decreased concentrations of sample buffer was tested. The recommended input of 1µL DNA with 9µL of sample buffer was altered such that 3µL and 5µL DNA was added to 70% and 50% sample buffer respectively.

Figure 3.1 WGA reactions with Varied Buffer Concentrations

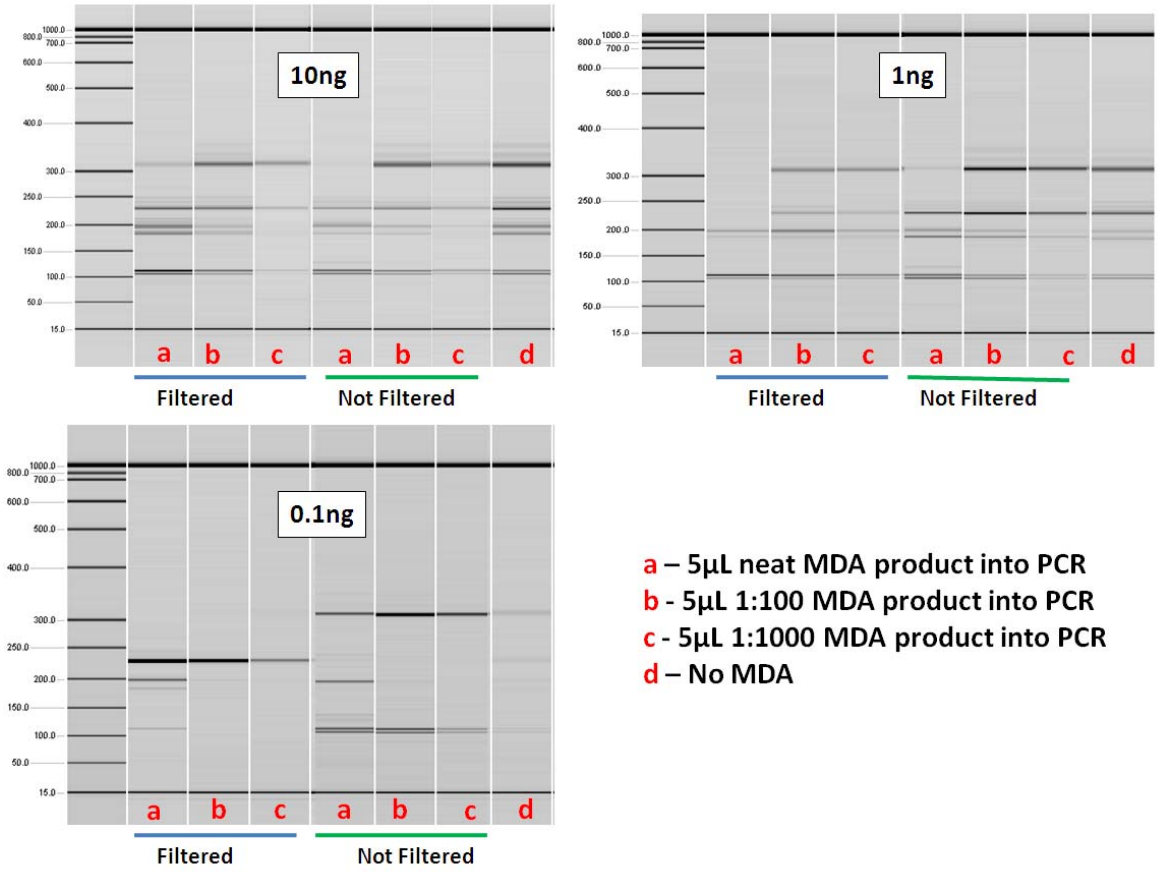


a – No MDA
b - 1µL DNA + 9µL sample buffer
c - 3µL DNA + 7µL sample buffer
d - 5µL DNA + 5µL sample buffer

The results indicated that the sample buffer could be reduced to 50% reaction volume with no significant loss of WGA product. However with degraded DNA WGA seems to perform slightly better with 90% sample buffer.

HMW DNA although showing high yields after WGA does not result in complete profiles using the STR 4-plex suggesting that there may be PCR inhibitors carried through from the WHA reaction. In order to remove any potential inhibitors the WGA reaction products were filtered prior to PCR using the STR 4-plex.

Figure 3.2 WGA reactions Filtered Prior to STR PCR Analysis
Analysis of WGA reactions of 10ng, 1ng and 0.1ng template DNA filtered prior to STR PCR.



The results indicate that in general no improvement in STR results were obtained by filtering WGA products prior to PCR. As the WGA template amounts decrease, more complete STR profiles are seen without filtration than those which were filtered prior to WGA. More specifically, a 1:100 dilution of WGA product prior to PCR for input amounts ≥1ng and neat WGA product input for those <1ng yielded best results and therefore would be recommended as a guide for future amplification. As expected the stochastic effects are amplified with 0.1ng WGA template. However an overall increase in product after WGA is seen.

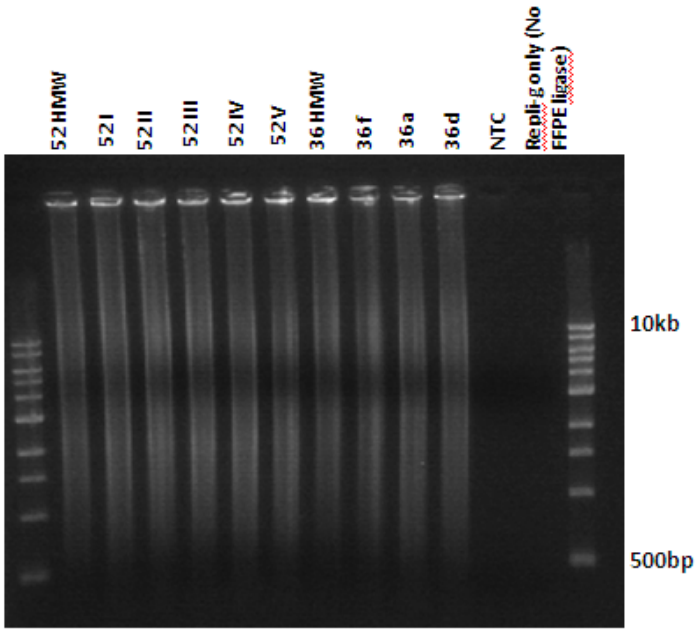
The filtration step will potentially result in inconsistent volume yield from filters, loss of template, extra sample handling and therefore increased risk of contamination. For these reasons ethanol precipitation of the WGA reaction was investigated as an alternative to filtration. The results showed that ethanol precipitation did not improve amplification and, in fact, more PCR product was detected without the ethanol precipitation cleanup step (data not shown).

These samples will be further analysed by capillary electrophoresis and STR PCR.

3.2.2 FFPE Repli-g

Figure 3.3 FFPE WGA of Degraded DNA Samples

Agarose gel electrophoresis of FFPE reactions on degraded DNA sample 080052 I-VI and sample 080036 a, d and f both shown in Figure 1.4.



FFPE Repli-g Product

These samples have been amplified with PowerPlex ESI-16 (Promega) and Y-filer and will be analysed using the AB 3130 Genetic Analyser.

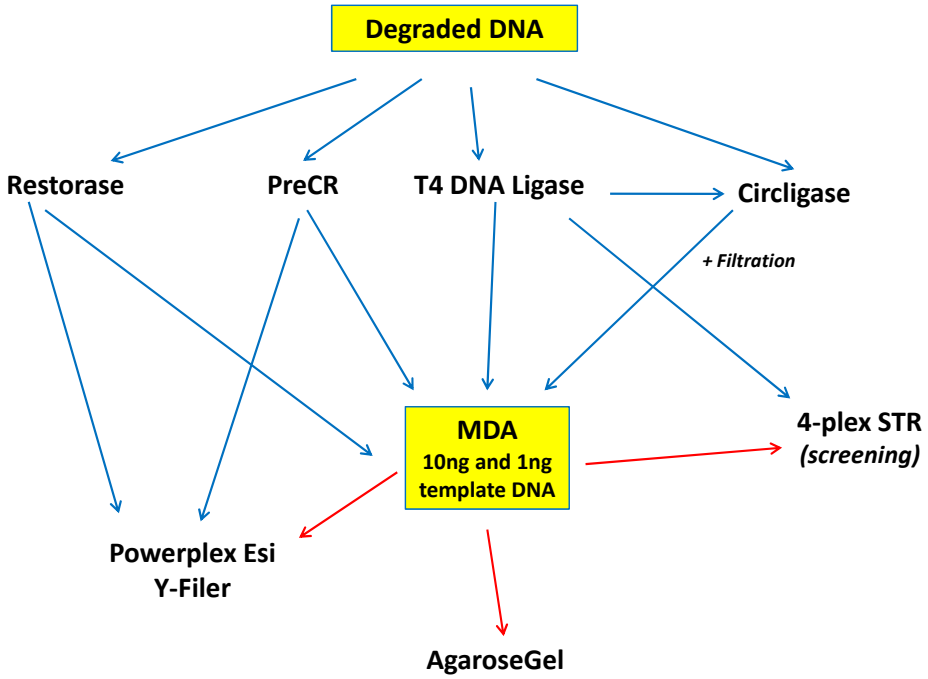
4. DNA REPAIR

DNA repair methods have been attempted in an effort to improve DNA typing success. Approaches that have been tried include

- T4 ligase +/- WGA
- T4 + CircLigase + WGA
- CircLigase + WGA
- Restorase
- PreCR

This repair was conducted on highly degraded samples at ~1ng DNA. A schematic of the repair methods attempted is shown in Figure .

Figure 4.1 Schematic of DNA Repair Experimental plan



4.1 Methods

4.1.1 T4 DNA Ligase Linear Ligation

T4 DNA ligase (Invitrogen) was used to ligate degraded DNA template (100ng and 10ng/ reaction). 10µL reactions were performed using 5U T4 Ligase/reaction in a 1x T4 Ligase Reaction buffer. Reactions were incubated at 14°C for 18hours.

- *Variables investigated: 1U, 3U, 5U and 10U ligase/reaction*

4.1.2 CircLigase Circularisation

CircLigase II enzyme (EPICENTRE, Madison, Wisconsin), a ssDNA ligase, was used to circularise single stranded DNA fragments. Double stranded degraded DNA or T4 treated samples (1ng and 10ng) were denatured by heating to 95°C for three minutes and snap cooled on ice. Reactions were performed with 50U of enzyme,1X CircLigase II reaction buffer and 2.5mM $MnCl_2$ in a 10µL reaction volume. Reactions were incubated at 60°C for one hour. CircLigase enzyme was inactivated by heating to 80°C for 10 minutes. Maximal volume of T4 product (6-8µL) was added to the CircLigase reaction. A 55-base oligodeoxynucleotide positive control (provided in the kit) was included.

- *Variables investigated:* *Addition of 1M Betaine per reaction*
  Ihr versus 16-20hr incubation
  PNK treatment prior to CircLigase
  Filtration/cleanup using spin columns prior to WGA

4.1.3 PreCR

Artificially degraded DNA (10 and 1ng) was added to 1X ThermoPol buffer, 100µM dNTPs and 1X NAD+ to a total reaction volume of 24.5µL. PreCR repair mix (New

England BioLabs) was then added (0.5µL) and incubated at 37°C for 20 minutes. 10% of the PreCR reaction volume (2.5 µL) was added to downstream STR genotyping.

- *Variables investigated:* *Incubation at 37°C for 20 minutes or 4°C overnight*
  50 µL versus 25 µL reaction volumes
  Input amount of reaction into downstream PCR (10% versus maximal volume)

4.1.4 Restorase® DNA Polymerase

Degraded DNA (5ng and 50ng) was incubated in Restorase reaction buffer, 200µM dNTP mix and 0.2µL Restorase® DNA polymerase (Sigma) in a 10µL reaction volume. Samples were incubated at 37°C for 10 minutes, 72°C for 5mins and denatured at 94°C for 30secs. 5µL of the reaction volume was added to downstream WGA or PCR.

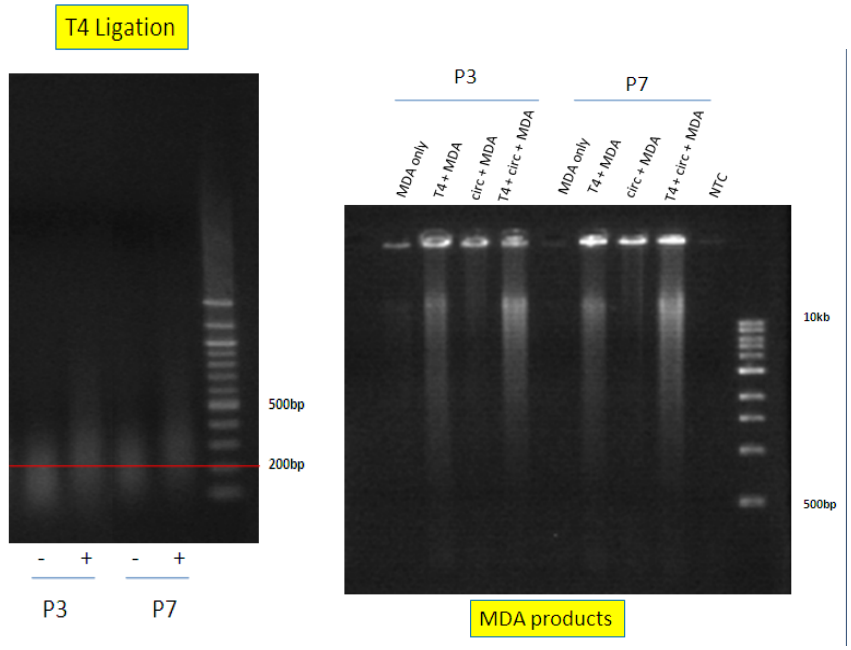
4.2 Results and Discussion

4.2.1 T4 DNA Ligase Linear Ligation

T4 DNA ligase ligates double stranded DNA fragments with either cohesive or blunt ends that have 3'-hydroxyl and 5'-phosphate ends. Attempts were made to increase the template length using T4 DNA ligase prior to PCR or WGA. The ligated degraded DNA samples were electrophoresed on an agarose gel to assess changes in DNA fragment sizes. The average length of DNA fragments had marginally increased (see Figure 3.1).

Figure 4.1. T4 Ligase Repair

A. Agarose gel electrophoresis of samples P3 and P7 before (-) and after (+) ligase treatment.
B. Agarose gel electrophoresis of samples P3 and P7 after ligase (T4), CircLigase (circ) or T4 and Circligase (T4 + circ) treatment following whole genome amplification (WGA).



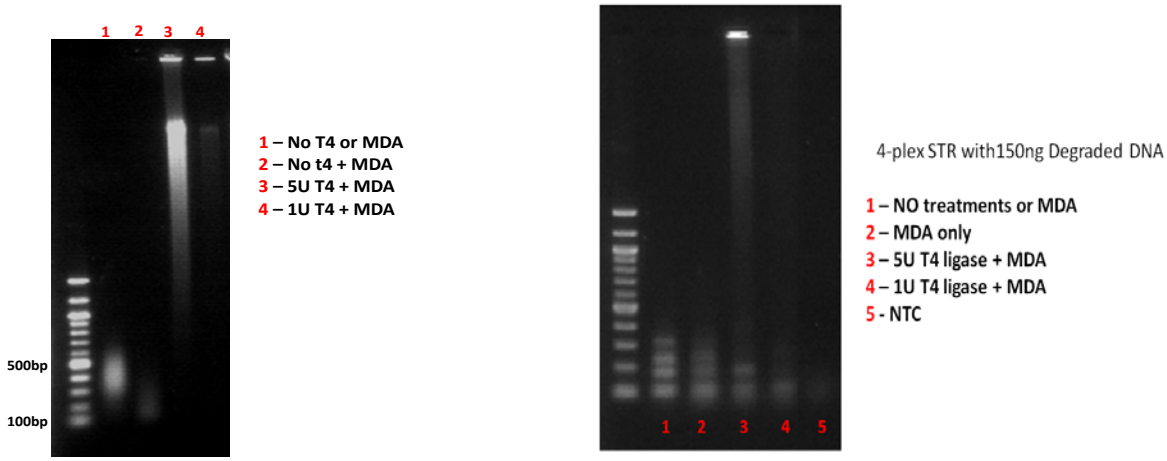
Although only a slight increase in template length after T4 is seen, some HMW product is yielded with WGA after T4 ligase treatment. However, this may be explained by the

possibility that with such large amounts of degraded DNA (>10ng) a small percent of larger fragments are still available as template for WGA. No successful yield of HMW product by WGA has been observed with smaller template amounts of degraded DNA.

Increase in T4 DNA ligase concentration from the recommended amount of 1U/reaction to 5U/reaction improved the product yield after WGA with 150ng (Figure 3.2). All subsequent T4 treatments were therefore carried out with 5U T4 DNA ligase/reaction.

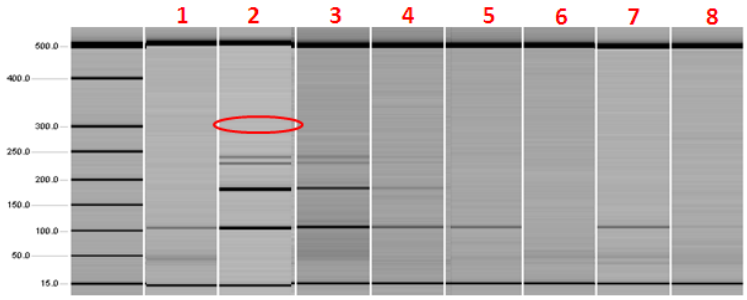
Figure 4.2. T4 Ligase Enzyme Level

A. Agarose gel comparison of T4 ligase repair using 5U (lane 3) and 1U (lane 4)
B. STR PCR results of T4 ligase repair using 5U (lane 3) and 1U (lane 4)



Downstream STR 4-plex PCR (Figure 3.2B) showed a loss of loci amplification despite ample HMW product seen on the gel post-WGA (Figure 3.2A). PCR inhibition due to WGA reagents may be the cause of this result. Dilution of the WGA product (1:100- 1:1000), spin columns and ethanol precipitation were attempted to overcome the possible inhibition. None of these treatments resulted in any significant improvement.

Figure 4.3. WGA Reaction Clean-up



1 – MDA only (150ng)
2 – NO treatments, No MDA (150ng)
3 – T4, No MDA (150ng)
4 - T4, No MDA (1.5ng)
5 – T4 + filtered + MDA (150ng)
6 - T4 + filtered + MDA (1.5ng)
7 - T4 - filtered + MDA (150ng)
8 - T4 - filtered + MDA (1.5ng)

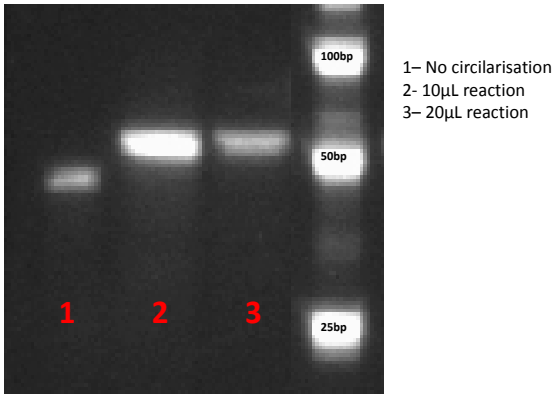
In order to maximise the amount of T4 treated DNA template in the subsequent WGA reaction and also to assess whether any carry-over reagents from the T4 incubation was

inhibiting the WGA, filter spin columns were used. The 4-plex STR results in Figure 3.3 indicate that the most complete PCR results from degraded DNA are obtained without WGA. Three out of four loci were successfully amplified from samples without any treatment as well as from the T4 treated samples without WGA amplification. However, amplification is not balanced at 1.5ng showing a significant decrease in amplification of the larger locus (TPOX at ~250bp). Filtration between the T4 ligase and WGA steps did not improve the STR results. There was inconsistent volume yield, loss of template, extra sample handling and increased risk for contamination as a result of the filtration step.

4.2.2 Circularisation

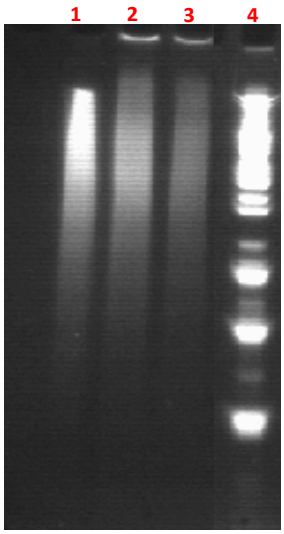
The manufacturer's recommended 20ul reaction volume was reduced to 10ul in order to add maximal CircLigase product to subsequent WGA reaction. Figure 3.4 shows improved results with the reduced volume reaction.

Figure 4.4. CircLigase Reaction Volume
The CircLigase reactions of 10uL (lane 2) or 20uL (lane 3) using the single stranded oligo provided in the manufacturer kit were analysed by polyacrylamide gel electrophoresis (PAGE).



The success of the circularisation of degraded samples has been difficult to assess. To date an improvement in product yield after WGA has been used as a guide. However this does not necessarily nor directly demonstrate that degraded fragments are circularising. The viability of the kit is assessed by visualising the single stranded +ve control oligo (55bp) included in kit via PAGE (Figure 3.4). A 'shift' in the smear which may indicate success of circularisation of degraded DNA prior to WGA cannot be seen (Figure 3.5).

Figure 4.5. CircLigase Reaction Volume
CircLigase reactions using degraded DNA were analysed by polyacrylamide gel electrophoresis (PAGE).

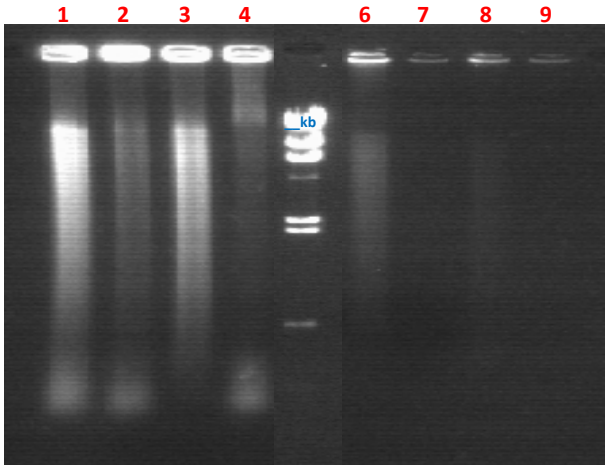


1 – degraded DNA – circligase
2 – degraded DNA +circligase (10ul reaction)
3 - degraded DNA +circligase (20ul reaction)
4 - Ladder

PAGE – denatured gel

Any improvement in circularisation has been measured by an increase in downstream WGA yield. CircLigase reactions incubated for one hour or 20 hours were clompared. The recommended 1hr CircLigase incubation was found to produce an equivalent yield to 20hrs for 150ng degraded DNA after WGA. A slight improvement in yield was seen with the one hour incubation for 1.5ng input DNA after WGA (Figure 3.6).

Figure 4.6. CircLigase Reaction Volume
Agarose gel electrophoresis of WGA reactions of CircLigase reaction samples using 150ng or 1.5ng degraded DNA.

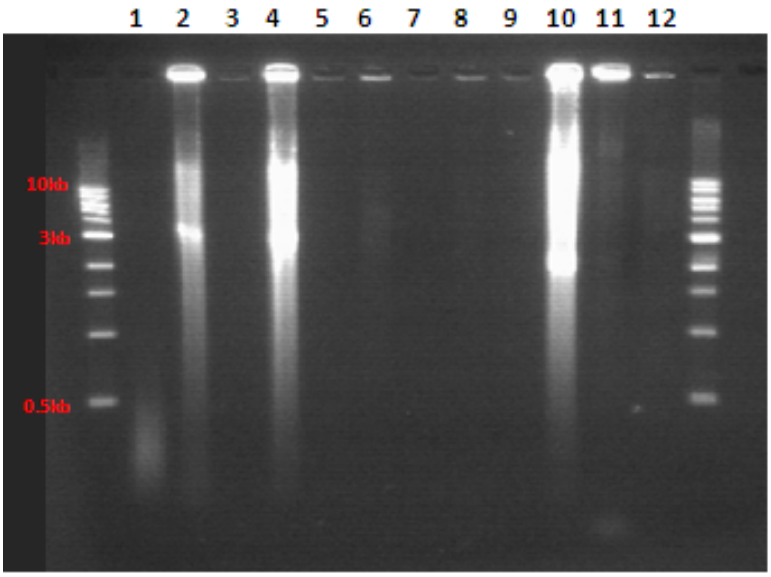


150ng
1 – 1hr circ + Filter + MDA
2 - 1hr circ - Filter + MDA
3 – 20hr circ + Filter + MDA
4 - 20hr circ - Filter + MDA

1.5ng
6 – 1hr circ + Filter + MDA
7 - 1hr circ - Filter + MDA
8 – 20hr circ + Filter + MDA
9 - 20hr circ - Filter + MDA

Results also show that filtration after CircLigase prior to WGA increased yield (fig. above and below). It was thought that reagents from the CircLigase reaction were inhibiting the WGA reaction. To confirm this, HMW control DNA (10ng) was spiked with CircLigase product (lane 11, fig below) prior to WGA resulting in significant inhibition.

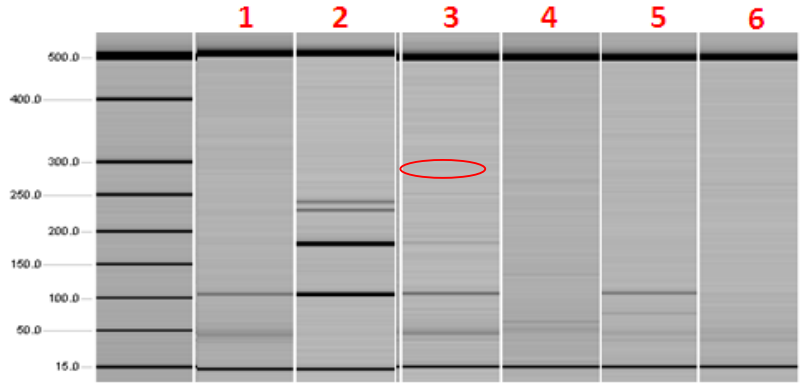
Figure 4.7. CircLigase Reaction Filtration Prior to WGA
WGA reactions of filtered CircLigase reactions of 100ng, 10ng, 1ng and 0.1ng degraded DNA.



4.2.3 T4 + CircLigase

The combination of initial linear ligation using T4 and subsequent circularisation in order to lengthen the circular template for WGA was tested. To date these experiments have not resulted in improved genotyping results.

Figure 4.8. T4 Ligase + Filtration + CircLigase treatment Prior to WGA
STR analysis of WGA reactions T4 ligse + CircLigase reactions of 150ng or 1.5ng degraded DNA.



1 – MDA only (150ng)
2 – NO treatments, No MDA (150ng)
3 – T4 - filtered + circligase + MDA (150ng)
4 - T4 - filtered + circligase + MDA (1.5ng)
5 - T4 + filtered + circligase + MDA (150ng)
6 - T4 + filtered + circligase + MDA (1.5ng)

The results shown in Figure 3.8 demonstrate a slight improvement in STR genotyping. One additional locus is amplified when filtration was omitted between T4 ligase and subsequent CircLigase treatment. However the fact that this is only seen with 150ng and not with smaller template amounts suggest that it may be more likely due to the loss of larger DNA fragments via filtration than removal of any inhibitors. Further investigation is required.

4.2.4 PreCR

Degraded DNA samples have been treated with PreCR plus and minus WGA. These samples have been amplified with PowerPlex ESI-16 (Promega) and will be analysed using the AB 3130 Genetic Analyser. If any show improvement in genotyping results they will be further analysed with Y-filer.

4.2.5 Restorase

Degraded DNA samples have been treated with Restorase plus and minus WGA. These samples have been amplified with PowerPlex ESI-16 (Promega) and will be analysed using the AB 3130 Genetic Analyser. If any show improvement in genotyping results they will be further analysed with Y-filer.

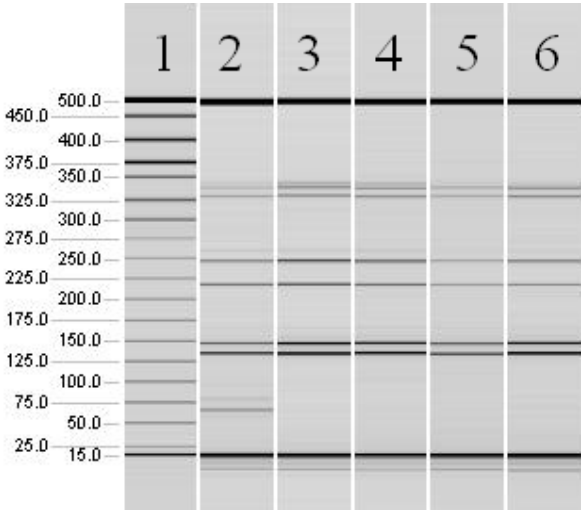
5. PCR AMPLIFICATION OF DEGRADED/LOW LEVEL DNA

5.1 Non-repaired DNA

As described in Section 3, 10 ng and 1 ng amounts of DNA were amplified using the RepilG whole genome amplification kit (Qiagen). Dilutions of the amplified DNA were subject to PCR analysis using a triplex STR PCR reaction. Results are shown in Figure .

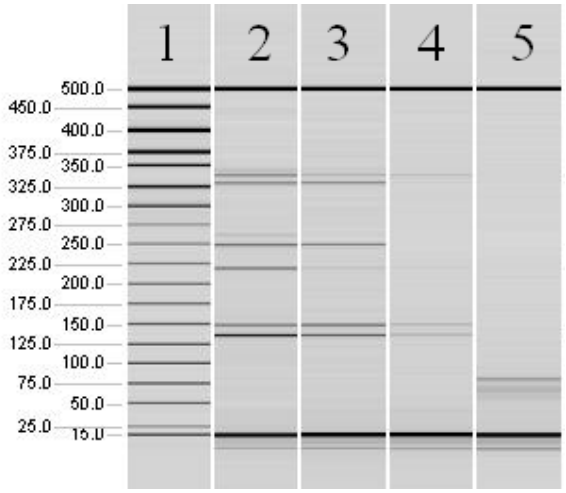
Figure 5.1 Whole genome amplification of 10ng (A. and B.) and 1ng (C.) of sample 83A.

A. 10ng of genomic DNA "83A" amplified by WGA and subject to PCR at neat, 1:10 and 1:100 dilutions



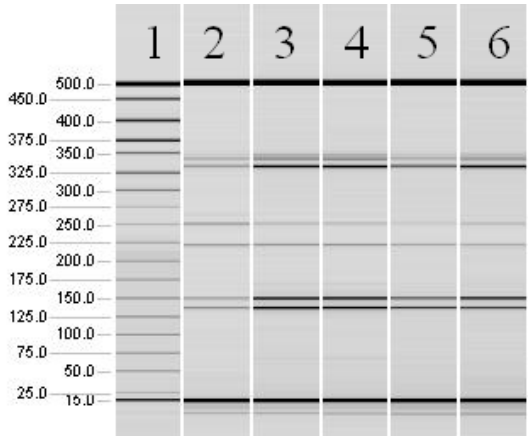
1. 15-450bp size marker
2. 83A 10ng without WGA
3. 83A 10ng with WGA (1:10 dilution)
4. 83A 10ng with WGA (1:10 dilution)
5. 83A 10ng with WGA (1:100 dilution)
6. 83A 10ng with WGA (1:100 dilution)

B. 10ng of genomic DNA “83A” amplified by WGA and subject to PCR at 1:1000 and 1:10,000 and 1:100,000 dilutions



1. 15-450bp size marker
2. 83A 10ng without WGA
3. 83A 10ng with WGA (1:1000 dilution)
4. 83A 10ng with WGA (1:10 000 dilution)
5. 83A 10ng with WGA (1:100 000 dilution)

C. 1ng of genomic DNA “83A” amplified by WGA and subject to PCR at neat, 1:10 and 1:100 dilutions



1. 15-450bp size marker
2. 83A 1ng without WGA
3. 83A 1ng with WGA (1:10 dilution)
4. 83A 1ng with WGA (1:10 dilution)
5. 83A 1ng with WGA (1:100 dilution)
6. 83A 1ng with WGA (1:100 dilution)

Degraded DNA samples were subject to WGA reactions were10 ng and 1 ng amounts of DNA were amplified using the RepilG whole genome amplification kit (Qiagen). Dilutions of the amplified DNA were subject to PCR analysis using a triplex STR PCR reaction. Results are shown in Figure .

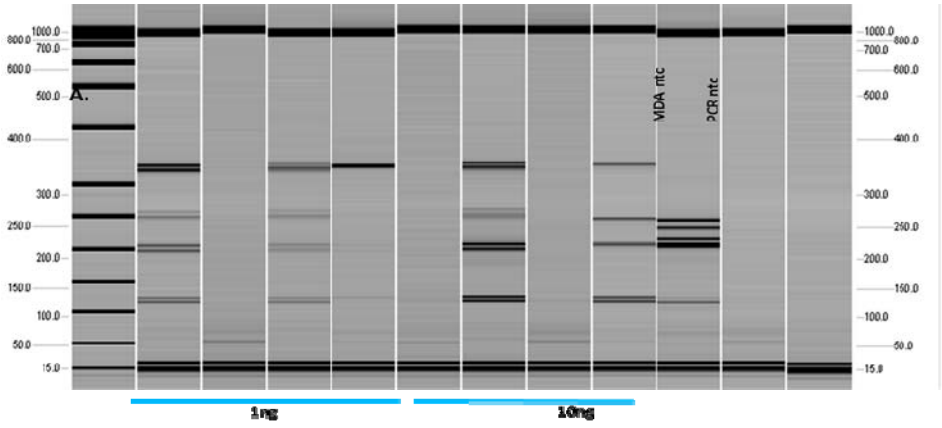
Samples were also analysed using the AmpFlSTR® Profiler Plus® PCR Amplification kits (Applied Biosystems) for STR genotyping. PCR was performed in 25µL reaction volumes on a GeneAmp 9700 thermocycler (Applied Biosystems) with cycling protocols as per kit manufacturer instructions. Electrophoresis was performed on a 3130 Genetic Analyser (Applied Biosystems). Samples were prepared for fragment analysis as per AmpFlSTR® Profiler Plus® PCR Amplification kit recommendations. Data analyses were performed using GeneMappper ID v 3.2.1 software (Applied Biosystems) with a 50 relative fluorescence units (RFU) peak amplitude threshold for all dyes.

5.2 Repaired Degraded DNA

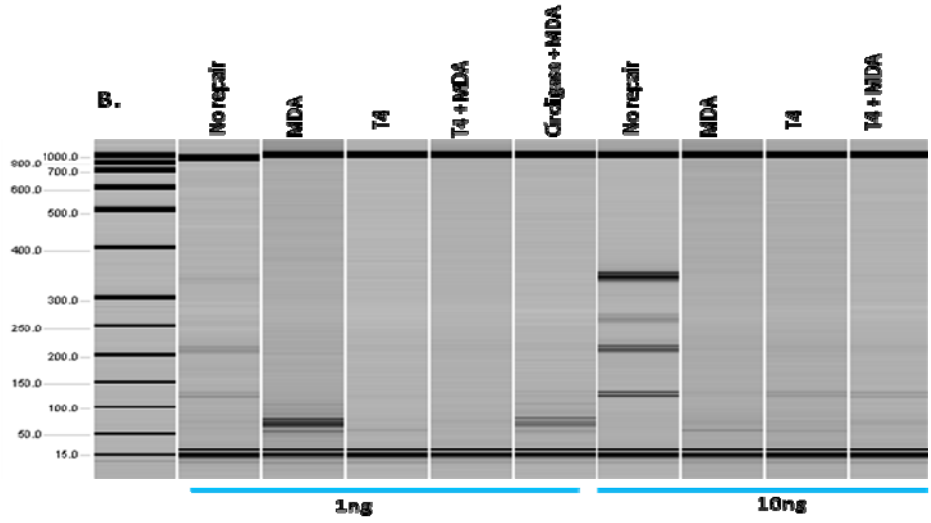
Repair treatment samples described in Section 4 have been analysed with and without WGA and have then been amplified with the STR quadruplex prior to possible downstream PowerPlex ESI-16 (Promega) and Y-filer STR typing.

Results from the STR quadreplex shown in Figure 5.2 show no improvement with the repair treatments.

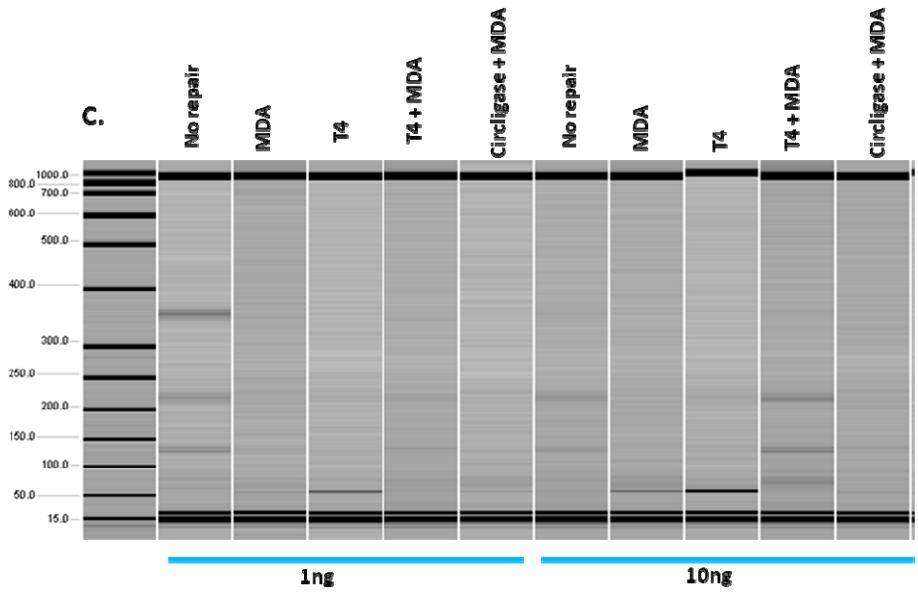
Figure 5.2 STR Quadruplex results with repair treatments to moderately (A.) highly (B.) and extensively (C.) degraded DNA.



Sample I – Moderately Degraded



Sample III – Highly Degraded



Sample VI – Extremely Degraded

Table 5.1 Results of PowerPlex ESI-16Analysis to date.

9/08/2010																
Sample = 83A																
SAMPLE	AMEL	D3S1358	D19S433	D2S1338	D22S1045	D16S539	D18S51	D1S1656	D10S1248	D2S441	THO1	VWA	D21S11	D12S391	D8S1179	FGA
10ng no WGA	Unable to discern genotype due to excessive amounts of artefacts (stutter/pull up) caused by sample overload															
10ng + WGA 1:10	Unable to discern genotype due to excessive amounts of artefacts (stutter/pull up) caused by sample overload															
10ng + WGA 1:100	X,Y	16,16	12,15.2	17,23	15,16	13,13	10,18	15,15	15,16	10,15	9,9	13,16	30,32.2	22,22	10,17	18,21
1ng no WGA	X,Y	16,16	12,15.2	17,23	15,16	13,13	10,18	15,15	15,16	10,15	9,9	13,16	30,32.2	22,22	10,17	18,21
1ng + WGA 1:10	Unable to discern genotype due to excessive amounts of artefacts (stutter/pull up) caused by sample overload															
1ng + WGA 1:100	X,Y	16,16	12,15.2	17,23	16,16	13,13	10,18	15,15	15,16	10,15		13,16	30,32.2	22,22	10,17	18,21
Positive Control 9947A	X,X	14,15	14,15	19,23	11,14	11,12	15,19	18.3,18.3	13,15	10,14	8,9.3	17,18	30,30	18,20	13,13	23,24
PCR NTC	No evidence of contamination															

12/08/2010															
PowerPlex ESI 16 on Degraded DNA															
SAMPLE	AMEL	D3S1358	D19S433	D2S1338	D22S1045	D16S539	D18S51	D1S1656	D10S1248	D2S441	THO1	VWA	D21S11	D12S391	D8S1179	FGA
P	X,X	15,17	14,16	17,24	11,16	12,14	14,17	14,15.3	13,14	13,14	9,9.3	18,19	29,31.2	18,19	12,16	19,21
P2	X,X	15,17	14,16	17,24	11,16	12,14	14,17	14,15.3	13,14	13,14	9,9.3	18,19	29,31.2	18,19	12,16	19,21
P2 1:10	X,X	15,17	14,16	17,24	11,16	12,14	14,17	14,15.3	13,14	13,14	9,9.3	18,19	29,31.2	18,19	12,16	19,21
P3	X,X	15,17	14,16	17,24	11,16	12,14	14,17	14,15.3	13,14	13,14	9,9.3	18,19	29,31.2	18,19	12,16	19,21
P3 1:10	X,X	15,17	14,16	17,24	11,16	12,14	14,17	14,15.3	13,14		9,9.3	18,19	29,31.2	18,19	12,16	19,21

120	X	15,17				12,14					9.9.3	18,19			12,16	
120 1:10		9				14					9,9.3				12,16	
KE	X,Y	14,15	14,14	20,24	15,16	12,13	13,17	12,15	13,15	10,14	9,9.3	17,18	28,29	19,20	14,14	23,24
KEE	X,Y	?	14,14	20,24	15,16	?	?	12,15	13,15	10,14	9,9.3	17,18	28,29	19,20	?	23,24
KEE 1ng	X,Y	14,15	14,14	20,24		12,13	13,17	12,15	13		9,9.3	17,18	28,29	19	14,14	23,24
b1											7,9.3				11,12	
b4	X,Y					12					7,9.3				11,12	
09037 Degraded	X,Y	15,16	12,14	20,23	11,15	12,13	14,15	11,15	12,15	12,14	6,6	16,16	32.2,33.2	19,22	13,15	23,24
9037	X,Y	15,16	12,14	20,23	11,15	12,13	14,15	11,15	12,15	12,14	6,6	16,16	32.2,33.2	19,22	13,15	23,24
NTC	No evidence of contamination															
Positive Control 9947A	X,X	14,15	14,15	19,23	11,14	11,12	15,19	18.3,18.3	13,15	10,14	8,9.3	17,18	30,30	18,20	13,13	23,24
Note: "?" Indicates unable to discern locus due to excessive artefacts (stutter/pull up) casued by sample overload																

18/08/2010																
PowerPlex ESI 16 on Degraded DNA																
SAMPLE	**AMEL**	**D3S1358**	**D19S433**	**D2S1338**	**D22S1045**	**D16S539**	**D18S51**	**D1S1656**	**D10S1248**	**D2S441**	**THO1**	**VWA**	**D21S11**	**D12S391**	**D8S1179**	**FGA**
48 HMW 1ng	X,Y	14,16	14,15.2	16,25	16,17	12,12	14,14	11,15	14,14	10,11	7,9.3	18,19	29,30.2	18,22	11,12	21,24
48a 10ng	X,Y	14,16	14,15.2	16,25	16,17	12,12	14,14	11,15	14,14	10,11	7,9.3	18,19	29,30.2	18,22	11,12	21,24
48a 1ng	X,Y	14,16	14,15.2			12,12	14,14				7,9.3	18,19	29		11,12	21,24
48b 10ng																
48b 1ng																
a1 10ng	X,Y	14,16	14,15.2	16,25	16,17	12,12	14,14	11,15	14,14	10,11	7,9.3	18,19	29,30.2	18,22	11,12	21,24
a1 1ng	X,Y	14,16	14,15.2	16,25	16,17	12,12	14,14	11,15	14,14	10,11	7,9.3	18,19	29,30.2	18,22	11,12	21,24
a3 10ng	X,Y	14,16	14,15.2	16,25	16,17	12,12	14,14	11,15	14,14	10	7,9.3	18,19	29,30.2	18,22	11,12	21,24
a3 1ng	X,Y	14,16	14,15.2	16,25		12,12	14,14				7,9.3	18,19	30.2		11,12	21,24
a5 10ng	X,Y	14,16	14,15.2	16,25	16,17	12,12	14,14	11,15	14,14	10,11	7,9.3	18,19	29,30.2	18,22	11,12	21,24

a5 1ng	X,Y	14,16	14,15.2	16,25		12,12	14,14	11,15	14,14	10	7,9.3	18,19	29,30.2	18,22	11,12	21,24
b30 10ng	X,Y	14,16	14,15.2	16	16,17	12,12	14,14	15	14		7,9.3	18,19	29		11,12	21,24
b30 1ng	X														13	
R HMW 1ng	X,Y	15,18	13,15	17,23	14,16	9,11	13,18	13,16.3	13,13	14,15	6,7	18,19	28,30	17.3,22	13,14	22,25
R30 10ng	X,Y					9					6,7				13,14	
R30 1ng															13	
R31 10ng															13	
R31 1ng																
NTC	X															
Positive Control 9947A	X,X	14,15	14,15	19,23	11,14	11,12	15,19	18.3,18.3	13,15	10,14	8,9.3	17,18	30,30	18,20	13,13	23,24

20/08/2010

PowerPlex ESI 16 on Degraded DNA

SAMPLE	AMEL	D3S1358	D19S433	D2S1338	D22S1045	D16S539	D18S51	D1S1656	D10S1248	D2S441	THO1	VWA	D21S11	D12S391	D8S1179	FGA
KE HMW	X,Y	14,15	14,14	20,24	15,16	12,13	13,17	12,15	13,15	10,14	9,9.9	17,18	28,29	19,20	14,14	23,24
R HMW	X,Y	15,18	13,15	17,23	14,16	9,11	13,18	13,16.3	13,13	14,15	6,7	18,19	28,30	17.3,22	13,14	22,25
R30 1ng No Repair	X										7					
R30 1ng T4 + WGA	X,Y										5,6				7	
NTC	No evidence of contamination															
Positive Control 9947A	X,X	14,15	14,15	19,23	11,14	11,12	15,19	18.3,18.3	13,15	10,14	8,9.3	17,18	30,30	18,20	13,13	23,24

24/08/2010

Sample = 08052

SAMPLE	AMEL	D3S1358	D19S433	D2S1338	D22S1045	D16S539	D18S51	D1S1656	D10S1248	D2S441	THO1	VWA	D21S11	D12S391	D8S1179	FGA

1ng High Molecular Weight	X,Y	15,16	12,13	17,23	14,17	11,12	13,18	14,15	14,14	10,11	8,9	14,19	28,31	17,22	13,14	19,24.2
1ng Degraded 1	X,Y	15,16	12,13	17,23	14	11,12	13,18	14,15	14		8,9	14,19	28,31	17	13,14	19,24.2
1ng Degraded 2	X,Y	15,16	12,13			11,12	13,18			10	8,9	14,17,19			13,14	19,24.2
1ng Degraded 3	X,Y						18				6,8,9	14			13,14	19,24.2
1ng Degraded 4	X,Y					12					8,9	14			13	19,24.2
1ng Degraded 5	X,Y										6,8,9					
1ng Degraded 6																
10ng Degraded 1	X,Y	15,16	12,13	17,23	14,17	11,12	13,18	14,15	14,14	10,11	8,9	14,19	28,31	17,22	13,14	19,24.2
10ng Degraded 2	X,Y	15,16	12,13	17,23	14,17	11,12	13,18	14,15	14,14	10,11	6,8,9	14,19	28,31	17,22	13,14	19,24.2
10ng Degraded 3	X,Y	15,16	12,13	17		11,12	13,18	14,15	14	10	6,8,9	14,19	28,31	17,22	13,14	19,24.2
10ng Degraded 4	X,Y	15,16	12,13	17,23		11,12	13,18	15	14		6,8,9	14,19	31		13,14	19,24.2
10ng Degraded 5		16													14	
10ng Degraded 6	X,Y	15,16				11,12	13		14		6,8,9	14,19			13,14	24.2

SAMPLE	AMEL	D3S1358	D19S433	D2S1338	D22S1045	D16S539	D18S51	D1S1656	D10S1248	D2S441	THO1	VWA	D21S11	D12S391	D8S1179	FGA
1ng High Molecular Weight	X,Y	16,17	13,14	17,19	15,16	11,12	12,16	11,15	13,15	11,15	7,9	17,18	29,33.2	18,23	13,14	22,22
1ng Degraded 1	X,Y					11,12	12,16				7,9	13,16, 17			10,13,14	
1ng Degraded 2	X,Y					11					7,8,9				13,14	22
1ng Degraded 3	X,Y										6,7,8					

10ng Degraded 1	X,Y	16,17	13,14	17,19		11,12	12,16	11,15	13		7,9	13,15, 16,17, 18	33.2	18	9,10,13,14, 15,16,17	17,18, 21,22
10ng Degraded 2	X,Y	16,17	13,14	17,19		11,12	12,16	11,15	13,15		6,7,8,9	17,18	29	18,23	13,14	22,22
10ng Degraded 3	X,Y	16				11,12					6,7,8,9	17,18			13,14	22
Positive Control 9947A	X,X	14,15	14,15	19,23	11,14	11,12	15,19	18.3,18.3	13,15	10,14	8,9.3	17,18	30,30	18,20	13,13	23,24