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EVALUATION OF GENOMIPHI WHOLE GENOME AMPLIFICATION KIT FOR USE IN LOW COPY NUMBER FORENSIC CASES

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CHAPTER I

INTRODUCTION

Forensic science has benefited from testing biological material since the establishment of ABO blood typing in 1905. Since that time, new technologies and tests have been evaluated, implemented and also replaced. One attribute common to most new tests is the ability to decrease the amount of biological material needed for testing. DNA testing has allowed evidence to be associated with crimes and individuals with minimal amounts of starting material. Mainstream DNA testing began with the introduction of the restriction fragment length polymorphism (RFLP) technology (1). Although a very discriminating test, RFLP required high molecular weight and high quality DNA, 50 to 500 nanograms of DNA (2), an amount that is not always found in evidentiary samples. High molecular weight DNA is typically only available from large sources of biological material, blood and large cellular masses. RFLP was replaced with polymerase chain reaction (PCR) based testing during the 1990's (1). PCR methodology doesn't require the high quality molecular weight DNA used by RFLP. DNA sections targeted by most PCR primers are under 400 bases in length. Such small amplicons allow for DNA to be useful with most cases. Current PCR technology allows for the reproducible analysis of evidence containing as little as 250 picograms of DNA, 35 diploid cells. Results are obtained below this level, but due to stochastic amplification effects, accuracy can be problematic when analyzing samples from this low copy number (LCN) DNA. If there is

not enough DNA to amplify, then the analysis of evidence may be inconclusive, have very little weight, or be misinterpreted. There is, however, a strong desire to test evidence containing LCN DNA for investigative purposes, cold cases, and post-conviction testing.

While DNA of the quality and quantity needed for RFLP is still being left at crime scenes, law enforcement agencies wish to take advantage of the evolution of forensic science. There are methods proposed that allow an analyst to obtain DNA profiles from new types of evidence containing minimal amounts of biological material, such as minute blood stains, fingerprints, differential extractions, etc. Lowe *et al.* (3) reported that it is common for the Forensic Science Service (FSS) to recover DNA from a swabbing of a surface that was only touched. While this ability greatly aids law enforcement, there are some limitations. Primarily, how much of an area needs to be touched to yield a full short tandem repeat (STR) profile? Will a fingerprint do? Is a palm print required? So while contact with an object does deposit DNA, the question is whether or not enough DNA was deposited to obtain a genetic profile. No definite answer can be given to these questions since different individuals have varying propensities on the amount of DNA they may shed when making contact with an object (4). While the minimum amount of contact area needed for testing cannot be quantified, the amount of DNA recovered from the swab can be. Quantitation of contact areas provides a means to evaluate the DNA recovered from them. These samples may be considered low copy number (LCN) if the DNA obtained is very minimal. The definition

of LCN is not absolute, but a general consensus may be agreed upon that less than 100 picograms may be considered low copy number DNA (5 – 8).

DNA quantitation methods are problematic when dealing with LCN samples. Currently the most common quantitation method utilized in forensic labs is Applied Biosystems Quantiblot® slot blot hybridization. This method immobilizes the DNA to a nylon membrane and identifies the samples through use of a biotinylated probe and streptavidin – horseradish peroxidase enzyme complex in a colorimetric detection system. Visual comparisons are made against a user-made set of standards with total DNA yields of 10 ng to 0.15625 ng (2ng/μL – 0.03125ng/μL) (9). A problem, when trying to quantify LCN samples, is that it is common for the lower two standards not to be visualized when using colorimetric detection. Real-time PCR (Q-PCR) is replacing the Quantiblot system due to its lower sensitivity threshold (0.023 ng/μL), its processing time of thirty minutes, and the possibility of automation. It has been reported that Q-PCR quantitation often estimates DNA concentrations about 3.5 times higher than that reported by Quantiblot® (10). Also, it is common that when no results are seen with the Quantiblot® system and STR analysis is performed, a full profile can be obtained. As more labs move to a Q-PCR assay it is anticipated that they will obtain the ability to accurately determine the level of DNA with which they are dealing, especially at low levels.

When analyzing DNA typing results of LCN samples several problems may present themselves; namely, no profile or partial profiles, allelic imbalance, and allelic dropout. Complete profile loss is the first problem, which, while frustrating, does not

result in an error for the analysis. Partial profiles also result in no analysis errors; however, these results carry lower statistical weight than a complete profile. No error in analysis is made because the entire locus would be considered inconclusive if it is not seen and thus not considered part of the profile obtained. Depending on the number of loci that are lost, the weight of the evidence can drop so low that no valuable information is obtained. Matching results can be obtained with one locus, but if the allele present at that locus is found in 50% of the population, then the locus by itself provides no valuable information. Allelic imbalance can effect the interpretation of the results. If PCR amplification and analysis occurs with a hundred percent efficiency then the balance of allele heights will be equal. Heterozygous alleles should have similar relative fluorescent unit (RFU). Homozygous alleles should have RFU's double that of heterozygous alleles. Forensic laboratory interpretation guidelines should contain a measure of the acceptable level of heterozygous peak imbalance. When these values fall below the acceptable range, the sample may be considered a mixture of two individuals or inconclusive. Allelic dropout poses the greatest problem. When an entire locus drops out, this is a clear sign that information has been lost, since no alleles are seen for that locus. When a locus is truly homozygous then allelic dropout is not of concern because it will mimic locus drop out, providing no information. However, when a locus is truly heterozygous, allelic drop out will cause the locus to appear homozygous. This is where error in analysis can occur. If the evidentiary sample and the reference sample truly originated from the same source then the error that would occur would be a false exclusion based on DNA testing.

There is also the possibility of a false inclusion if alleles were lost from the evidentiary sample and a match was made to a reference sample.

Whole genome amplification (WGA) may provide the answer to these problems. Amplification of the whole genome has been used in medical diagnostics, research and other scientific settings where samples contain only enough DNA to perform one test (11). WGA extends the number tests possible to conduct on samples with very little amounts of DNA (12). This is achieved by pre-amplifying the entire genome, so more template copies will be present for downstream testing. Other methods of genome amplification exist but they require formation of a cell culture and time for culture growth or cloning of human DNA in plasmids of bacteria (12). Various methods have been proposed for current WGA and some of the major techniques are outlined below.

Linker-adaptor PCR utilizes restriction enzymes and adaptor links to amplify the whole genome. Restriction enzymes are used to digest the DNA and produce fragments with known sequences on the ends. Linkers are then ligated to the restricted ends of the fragments. Universal primers that are complementary to the linkers are used in a PCR reaction so that the digested fragments are targeted (13). Being able to utilize one primer is a major advantage to this reaction. Since no multiplex is required, PCR can occur more efficiently. Some disadvantages are that the digestion enzymes may cut within the section of DNA that is of interest. Also, the fragments produced by the enzymes may exceed the functionality of the polymerase being used (13). If the fragments are too long then the polymerase may not be able to replicate the entire fragment. The method also

results in the loss of data toward the end of the chromosomes since a linker can not be added to them.

Degenerate oligonucleotide primer (DOP) amplification is PCR-based and employs degenerate primers with fixed ends. This PCR-based amplification utilizes several low temperature annealings and extensions to allow for multiple binding sites. This is followed by raising the annealing temperature so specific priming only occurs at the fragments containing the first primer sequences (14). Some disadvantages of this technique are that the product generated never amplifies better than the starting material, usually about one-third as well. Possible reasons for this could be due to non-specific binding of primers and formation of non-specific DNA molecules which have no relation to the human genome. This could be problematic with later analysis. Another disadvantage is the large amount of input DNA required; about 40 ng is needed to yield accurate and reliable results (14).

Primer extension preamplification (PEP) is a PCR-based method that uses a collection of 15-base oligonucleotide primers in which any base can occupy any space. Theoretically the primer mix would contain about 1×10^9 (4^{15}) different primers (15). The PCR conditions consist of 50 cycles of 1 minute denaturation at 92°C, 2 minute annealing at 37°C, programmed ramping of 10 sec/degree to 55°C, and 4 minute extension at 55°C. Such a low annealing temperature with a slow ramp allows for relaxed annealing conditions to occur early in the cycle and more stringent annealing conditions towards the end (15). An advantage of this technique is the capability of amplifying DNA from a single cell. A disadvantage is the use of the PCR reaction. PCR

may introduce errors in the amplified DNA, through the denaturation and annealing cycles of PCR, resulting in incorrect analysis, especially when amplifying from a single cell (12).

Multiple displacement amplification (MDA) also utilizes random primers as PEP. One difference is the primers are only 6 bases in length not 15. The reaction is an isothermal reaction that is made possible by using bacteriophage Phi29 polymerase instead of *Taq* polymerase. Phi29 polymerase was originally chosen for its ability to perform strand displacement amplification for more than 70,000 nucleotides (12). PCR-based amplification can incorporate sequence errors and are limited to short segments of DNA (12); this is reduced when using Phi29 polymerase due to its 3'-5' exonuclease proofreading activity. This proofreading activity along with the polymerase's high fidelity gives amplification an error rate of 3×10^{-6} (mutations/nucleotide) compared with an error rate of 1×10^{-3} when AmpliTaq DNA polymerase is employed (11). Another problem with PCR-based WGA, reported by Dean *et al* (16), is that large variations can occur between markers during amplification due to the annealing and denaturation of primers. This can decrease the utility of these methods because loci may not be fully amplified causing information loss in downstream analysis.

When *Taq* polymerase encounters double stranded DNA during amplification it will destroy the encountered strand. When Phi29 polymerase encounters a new strand it will displace the strand and continue to do so during strand extension. MDA occurs by the annealing of random hexamer primers allowing Phi29 to initiate extension at a multitude of sites. Strand displacement occurs every time the polymerase encounters a

new primer (new strand). As the DNA strand is displaced it will open up single stranded template for new primers to bind to. This amplification, displacement, and subsequent amplification is what allows the reaction not to require cycling of temperatures as with PCR-based WGA. Figure 1 depicts how multiple displacement amplification takes place.

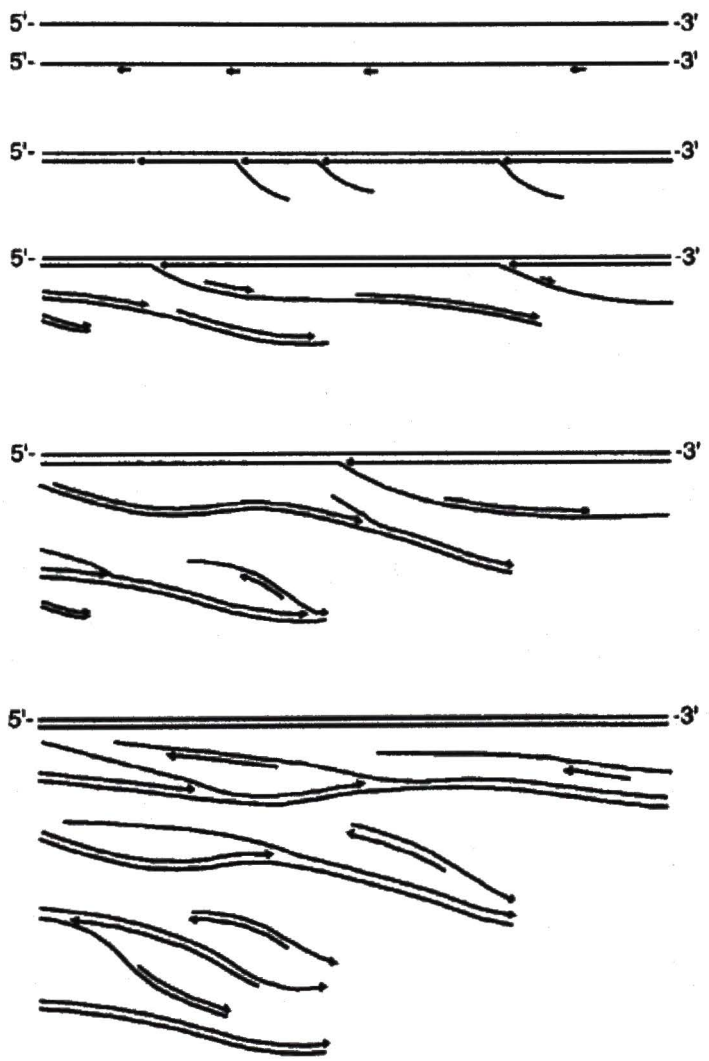

Figure 1: Representation of multiple displacement amplification. Initial annealing of random primers forms many events. As new strands are extended the Phi29 polymerase will encounter a primer and displace the DNA strand. As the strand is displaced more priming events can take place for new template to be amplified (17).

Amersham Bioscience produces the only available WGA kit, GenomiPhi DNA Amplification kit. It is a multiple displacement amplification reaction. The kit contains all reagents needed to perform WGA. Optimal amount of DNA input into the reaction is 1 ng or greater to yield 4 μg product. This amount is the optimal amount currently needed by PCR systems; however STR amplification requires the 1ng in 10 μL of sample extract. GenomiPhi requires 1 μL of sample extract, ten times the concentration of STR systems. The goal of this project was to evaluate the possible uses of the GenomiPhi DNA Amplification kit for use in the forensic community. The ability to amplify the entire genome without bias would benefit low copy number samples where there is little DNA to start with. One objective of the study was to determine the lower limit of input DNA into the GenomiPhi reaction. Input DNA varied from 10 ng to 7 pg. Secondly, input DNA was degraded to determine how the GenomiPhi kit will be affected by the input of less than pristine DNA. All samples were quantitated by PicoGreen assay, STR amplified with Profiler Plus and analyzed on ABI's 310 Genetic Analyzer. Samples were analyzed before and after WGA to determine under which circumstance better results were seen.

CHAPTER II

MATERIALS AND METHODS

Organic DNA Extraction

Modified from DNA Typing protocols (18):

Samples were collected from buccal swabs and blood stains. Blood was spotted onto cotton cloth and allowed to dry. Buccal swabs were taken from a male volunteer and allowed to dry. Cuttings from blood stain and buccal swabs were digested in 300 µL Stain Extraction Buffer (10mM Tris-HCl – 100mM NaCl – 10mM EDTA – 2%SDS) and 10 µL Proteinase K (Amresco, Cat#39450-06-6) at 56°C over night. DNA was purified with 300 µL Phenol: Chloroform: Isoamyl Alcohol (25:24:1, Amresco) and precipitated with 100% ethanol. Samples were resuspended in 200 µL of TE^{-4} (10 mM Tris-HCl – 0.1 mM EDTA).

DNA Dilutions

DNA extractions of buccal swabs and blood stains were quantified with the ABI Quantifiler kit to determine concentrations of stock solutions. DNA Dilutions prepared from stock solutions were quantified to verify DNA concentrations. The following dilutions were prepared from stock solutions of DNA in TE^{-4} buffer:

- 10 ng/µL
- 2 ng/µL
- 1 ng/µL
- 0.500 ng/µL
- 0.250 ng/µL
- 0.125 ng/µL

- 0.062 ng/μL
- 0.031 ng/μL
- 0.015 ng/μL
- 0.007ng/μL

Dilutions were quantified after preparation to verify concentration of DNA solutions.

DNA Quantitation

Quantitation of DNA extracts was carried out using real time PCR on an ABI 7000 Sequence Detection System using Quantifiler™ Human DNA Quantification kit (Applied Biosystems, Cat#4343895). Standards were produced by diluting the kit provided DNA standard (200 ng/μL) to the following concentrations:

- 50 ng/μL
- 16.67 ng/μL
- 5.56 ng/μL
- 1.85 ng/μL
- 0.62 ng/μL
- 0.21 ng/μL
- 0.069 ng/μL
- 0.023 ng/μL

Two microliters of extracted DNA was combined with 12.5 μL of reaction buffer and 10.5 μL of primer mix (prepared as a master mix). Sequence Detection Systems (SDS) Software v1.0 was setup to manufacture's specifications for human DNA quantitation (2). A blank plate template for absolute quantitation assay was opened. The Quantifiler Human and IPC dyes were added to the plate template. Standards were run in duplicate in wells A1 through B4. Thermocycling conditions were as follows:

- 95°C for 10 minutes
- 40 cycles
    - 95°C for 15 seconds
    - 60°C for 1 minute

Following WGA amplification quantitation of the product was carried out using the PicoGreen (Molecular Probes, Cat#P11495) assay. A working solution of PicoGreen reagent was prepared by diluting the reagent 1:100. Samples were prepared in a volume of 100 μL in TE^{-4}. One hundred microliters (100 μL) of the working solution was added to each sample for a final volume of 200 μL. Fluorescence was measured with the Hitachi FMBIOII. DNA quantities were determined by formation of a standard curve using known concentrations of lambda DNA.

DNA Degradation

DNA was experimentally degraded with DNase I (Invitrogen, Cat#18068-015). Degradation was carried out on ice to slow the kinetics of the enzymatic reaction. Test samples were degraded and visualized on agarose yield gel to determine optimum degradation conditions. Ten microliters of DNA extract, 20 ng/μL, was degraded in 10 μL PCR buffer (15 mM $MgCl_2$) with 2μL (1 unit/μL) of DNase I. The degradation reaction was stopped by the addition of 2 μL of 25 mM EDTA after 15 seconds and followed by heat inactivation at 65°C for 10 minutes. Degraded DNA was compared to non-degraded DNA using a 2% agarose (Amresco, Cat#9012-36-6) yield gel. DNA was stained with ethidium bromide and visualized with a UV transilluminator.

Whole Genome Amplification

Whole genome amplification was carried out with Amersham Biosciences GenomiPhi DNA Amplification kit (Amersham Bioscience, Cat#25-6600-01) following manufacture's guidelines (19). Reactions were set up using 1μL of DNA from the dilutions, 9 μL of sample buffer (50 mM Tris-HCl pH 8.2, 0.5 mM EDTA and random

hexamers), 9 µL of reaction buffer (dNTP's in proprietary buffer), and 1 µl of enzyme mix (Phi29 polymerase and random primers in proprietary buffer). Samples and sample buffer were combined and heat denatured for 3 minutes at 94°C then snapped cooled on ice. Reaction buffer and enzyme mix were combined into a master mix of which 10 µL was aliquoted per sample. WGA was carried out on a GeneAmp 9700 thermocycler (Perkin Elmer) with the following conditions:

- 9 cycles
    - 30° C for 60 minutes
    - 30° C for 60 minutes
- 65°C for 10 minutes (enzyme inactivation)
- 4°C hold

Each DNA dilution was amplified with the GenomiPhi kit four times, except the 10 and 2 ng/µL which were amplified three times. Non-degraded and degraded DNA samples were also amplified in triplicate.

STR Analysis

AmpFlSTR Profiler Plus kit (Applied Biosystems, Cat#4303326) was used to amplify nine short tandem repeat loci. Each reaction was carried out with approximately 1 ng of input DNA in 10 µL with final reaction volume of 25 µL. Fifteen (15) µL of master mix (10.5 µL of reaction buffer, 5.5 µL primer pairs, and 0.5 µl of AmpliTaq Gold (Applied Biosystems, Cat#N8080243)) was used per reaction. The following thermocycler conditions were used to amplify the STR loci:

- 95°C for 11 minutes
- 28 cycles

    - 94°C for 1 minute
    - 59°C for 1 minute
    - 72°C for 1 minute
- 60°C for 45 minutes
- 4°C hold

STR analysis was carried out on the ABI Prism 310 Genetic Analyzer (Applied Biosystems). One µL of sample was prepared with 10 µL of Hi-Di Formamide (Applied Biosystems, Cat#4311320) and 0.5 µL of GeneScan 500 ROX (Applied Biosystems, Cat#401734) internal lane standard. Performance optimized polymer 4 (Applied Biosystems, Cat#402838) was used to separate the STR fragments. Analysis was concluded with GeneScan 3.7 and Genotyper 3.7 software using an RFU threshold of 150.

CHAPTER III

RESULTS

DNA Extraction and Quantitation

DNA extraction from buccal and blood stains yielded varying amounts of DNA. Quantitation, by Q-PCR, of the buccal samples, B1 and B2, yielded 2.94 and 20.20 ng/μL of DNA respectively. The blood stains, BS1 and BS2, contained 16.88 and 4.35 ng/μl of DNA respectively. Dilutions were prepared in duplicate, one replicate from the buccal swab stock solutions and the other from the blood stain stock solutions. Quantities of DNA in the dilutions were determined by Q-PCR as outlined previously. Table 1 shows quantities of DNA in dilutions.

Table 1: Quantity of DNA in prepared dilutions.

Target Concentration	Stock Solution	Quantity (ng/μL)
1	B2	1.210
	BS1	1.180
0.500	B2	0.809
	BS1	0.609
0.250	B2	0.369
	BS1	0.292
0.125	B1	0.174
	BS2	0.234
0.062	B1	0.094
	BS2	0.088
0.031	B1	0.060
	BS2	0.070
0.015	62-B1	0.018
	62-BS2	0.030
0.007	31-B1	0.017
	31-BS2	0.015

Sample name indicates desired concentration of sample in ng/μL Samples were prepared from stock solutions listed. Dilutions of 0.015 and 0.007 pg were prepared from 0.062 and 0.031 dilutions.

The 10 and 2 nanogram dilutions were prepared from sample B2. Due to time limitations these dilutions were not quantified for this portion of the study and are based on the initial quantification of these samples. The reagent blank showed 2 pg/µl of DNA. Table 2 list the quantities of the dilutions used in the study.

Table 2: Sample quantities

Sample Name	DNA quantity (ng/µL)
A	10
B	2
C	1.180
D	0.609
E	0.234
F	0.094
G	0.060
H	0.030
I	0.015
Reagent Blank	0.002
Degraded	8
Non-degraded	8
WGA Positive	10

DNA Degradation

Test samples of 9947A DNA from a known source and concentration provided in Profiler Plus kit, were degraded to determine the optimal time for incubation with DNase I. One nanogram (1 ng) of DNA was incubated for 0 to 120 seconds. An agarose yield gel was used to visualize DNA degradation. DNA was not detected in any of the samples, including the 0 second incubation. It was determined that the quantity of DNA loaded into the agarose yield gel, 300 pg, was insufficient for visualization utilizing ethidium bromide.

The DNA degradation experiment was repeated using sample B2 due to its high concentration of DNA. Ten microliters of sample B2, approximately 200 ng of DNA,

was incubated for 30 seconds with 1μL of DNase I. The degraded DNA could not be seen on an agarose yield gel. Since partial degradation of the sample was wanted, the reaction was performed again. The incubation time with DNase I was reduced to 15 seconds. The degraded DNA bands appeared very similar to that of non-degraded DNA. The 15 second degradation sample was used for analysis by WGA.

Whole Genome Amplification and Quantitation

Whole genome amplification was carried out on the dilutions and the 15 second degraded DNA, as outlined in Table 2. Each sample was amplified four times, except sample A, B, degraded and non-degraded samples which were amplified 3 times.

Following whole genome amplification, quantitation was performed using PicoGreen and analyzed with Hitachi FMBIOII, Figure 2 and 3 show the results of the assay. Samples were quantitated using 1 or 2μL of WGA product. A standard curve was produced using lambda DNA ranging from 150 to 450 nanograms. Table 3 reports average quantities of DNA from WGA samples.

Table 3: Quantity of DNA produced by WGA

Sample	Average Quantity of DNA (ng/μL)	Std Dev	Total DNA (μg)
A	300.29	13.25	6.01
B	272.84	16.58	5.46
C	254.54	10.58	5.09
D	225.21	25.77	4.50
E	216.84	20.28	4.34
F	216.76	19.93	4.34
G	217.83	18.23	4.36
H	219.89	11.96	4.40
I	219.54	20.03	4.39
Reagent Blank	197.75	- - -	3.95
Degraded	3.37	10.08	0.07
Non-degraded	298.64	9.56	5.97
Positive	328.95	96.16	6.58

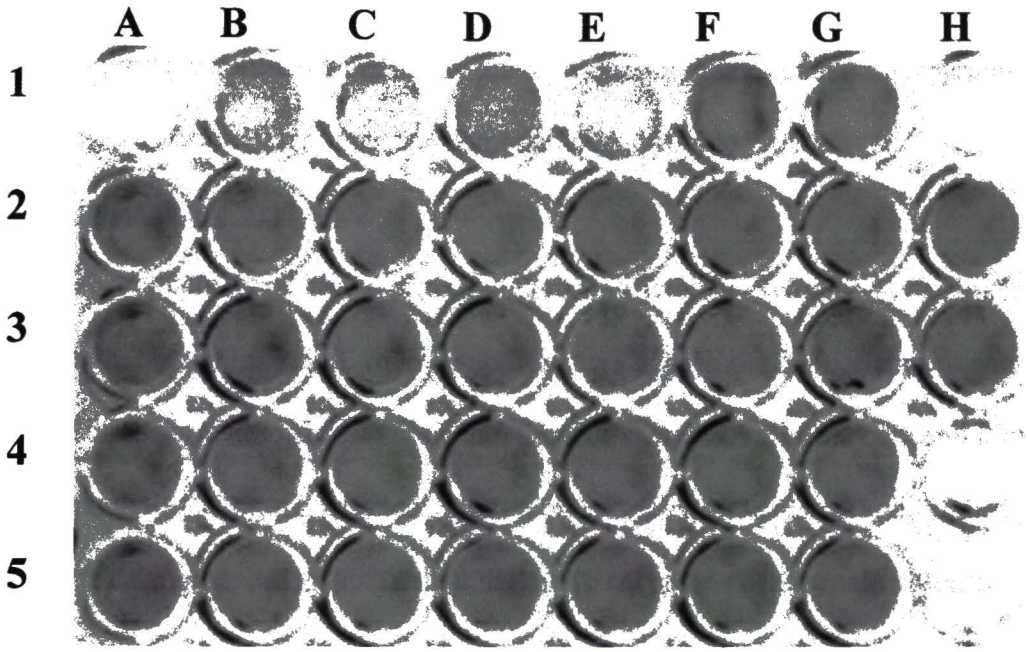

Figure 2: PicoGreen assay. Standard are in row 1, A-0 ng, B-100 ng, C-150 ng, D-200 ng, F-240 ng, and G-400 ng, E is an internal control. Columns, rows 2 – 5, are WGA of the following samples: A - C, B - D, C - E, D - F, E - G, F - G, and G - I. H2 is the reagent blank and H3 is the WGA positive control.

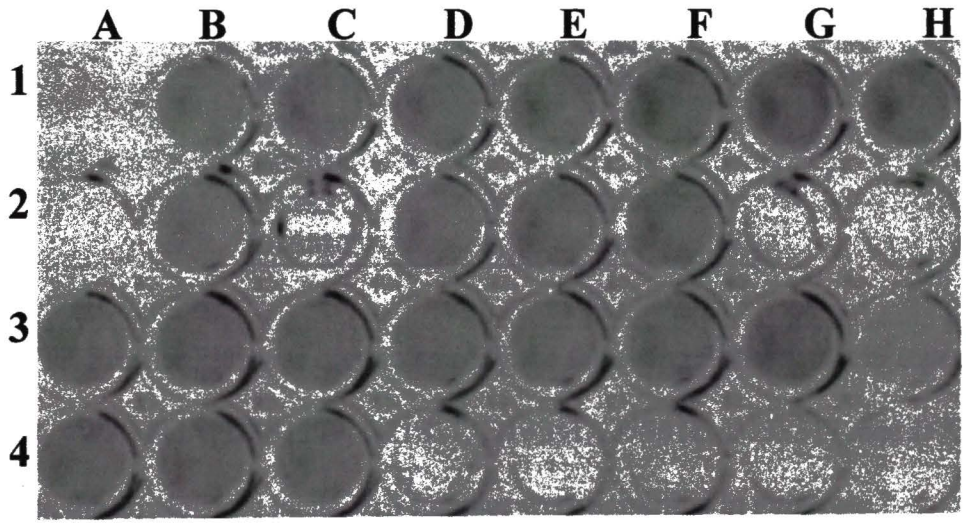

Figure 3: PicoGreen assay. Standards are in row 1, A-0ng, B-150 ng, C-200 ng, D-240 ng, E-300 ng, F-360 ng, G-400 ng, and H-480 ng. Sample A, 10 ng input DNA, are in A3, B3, and C3. Sample B, 2 ng input DNA, are in D3, E3, and F3. G3 is the WGA positive control. Wells A4 – C4 are the non-degraded sample. Wells D4 – F4 are the degraded sample. Row 2 contains standards not used in analysis

The WGA positive controls work as expected producing greater than 4 micrograms of DNA (200 ng/μL) from 10 ng of starting lambda DNA, provided in kit.

STR Amplification and Analysis

WGA products were diluted prior to PCR amplification. Samples were diluted by a factor of eight by combing 5 μL of WGA product with 35μL of dH_20. Samples were then diluted once more according to concentrations of samples with final concentrations of approximately 0.125 ng/μL targeted. Non-WGA samples were also diluted if they were above 0.200 ng/μL of DNA. Table 4 list the DNA profile of the male volunteer.

Table 4: Reference profile of volunteer

Locus	Alleles
D3S1358	17, 18
vWA	14, 17
FGA	19, 22
Amelogenin	X, Y
D8S1179	14
D21S11	27, 32.2
D18S51	13
D5S818	11, 14
D13S317	9, 11
D7S820	12

AmpFlSTR Profiler Plus was used to amplify the following 9 short tandem repeat loci: D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, and D7S820, along with the amelogenin locus. All samples were amplified in duplicate.

Non-WGA samples produced complete profiles from sample A through G and non-degraded for both amplifications. Sample H, with 300 pg of DNA into PCR, showed multiple extraneous peaks with similar relative fluorescent units (RFU) for the first amplification, the second amplification contained a complete profile with no extraneous

peaks. Both samples were prepared again from the same PCR amplifications and analyzed again, Figure 4. The same profiles were seen from the original amplifications. Sample I only produced a partial profile for both amplifications. The degraded DNA sample only contained the amelogenin locus.

Whole genome amplified samples from A through E and the non-degraded sample produced complete profiles for at least one amplification. The degraded sample contained the amelogenin locus and a few more alleles, 11 at D5S818 and 14 at D8S1179 for the first amplification and 14 at D5S818 for the second amplification. Several other alleles were seen below the 150 RFU threshold of the degraded sample. Table 5 lists the number alleles seen in the profiles for non-WGA and WGA samples. A complete profile contains 17 alleles. See Table 10 and 11 in appendix for detailed allele calls.

Table 5: Number of alleles present in sample profiles

	WGA		Non-WGA	
Sample	AMP 1	AMP 2	AMP 1	AMP 2
A	17	17	17	17
B	17	17	17	17
C	17	17	17	17
D	16	17	17	17
E	17	17	17	17
F	15	16	17	17
G	14	14	17	17
H	8	7	12	17
I	7	4	11	13
Reagent Blank	0	0	0	0
Degraded	4	3	2	2
Non-degraded	17	17	17	17
WGA Positive 1	0	0	NA	NA
WGA Positive 2	0	5	NA	NA

Numbers represent the number of alleles that correspond to the reference profile. Alleles were only called when their RFU value was above 150

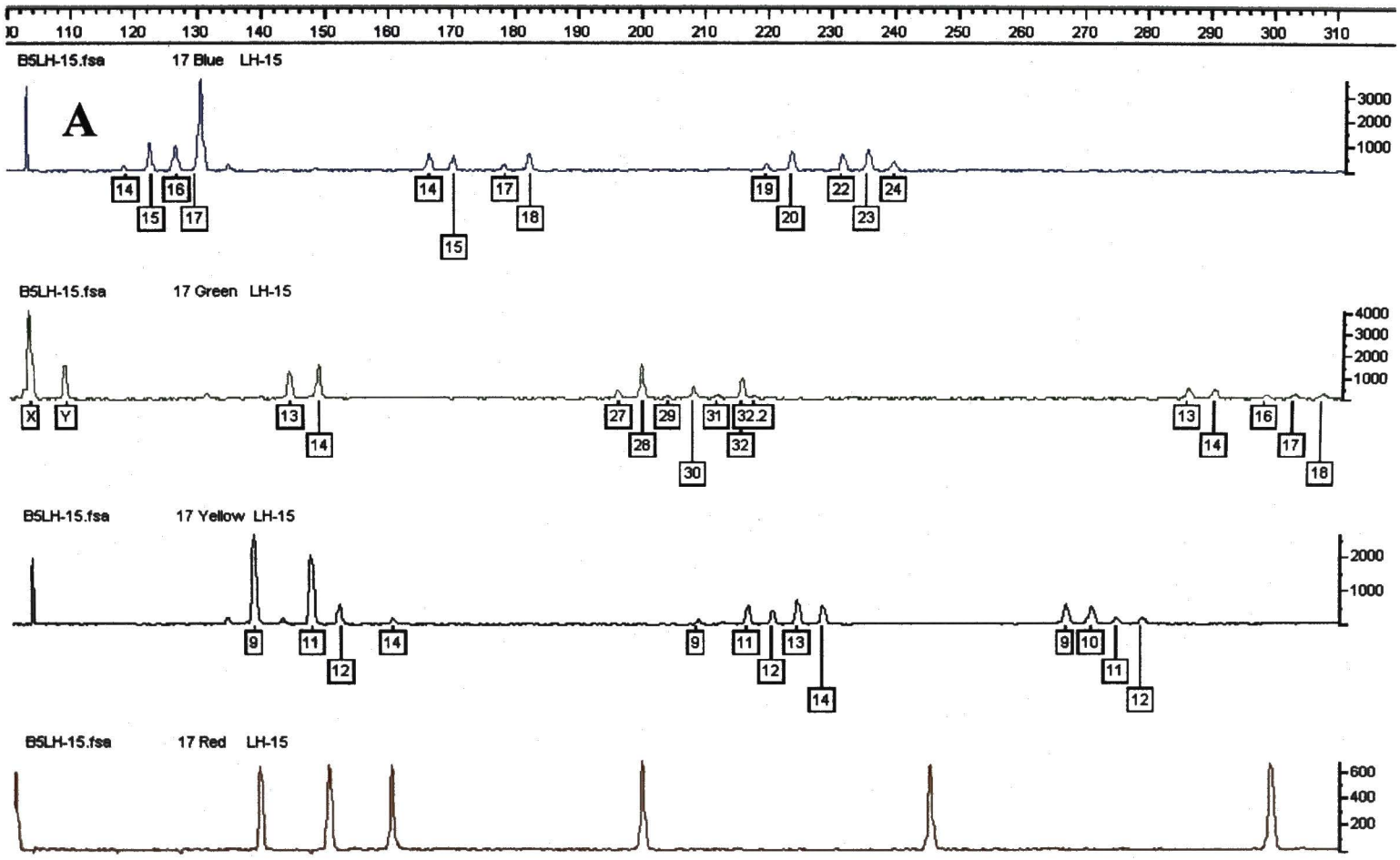

Figure 4A: Sample H. Second round of amplification to confirm profiles. A: Amplification 1, contains multiple alleles not associated reference profile.

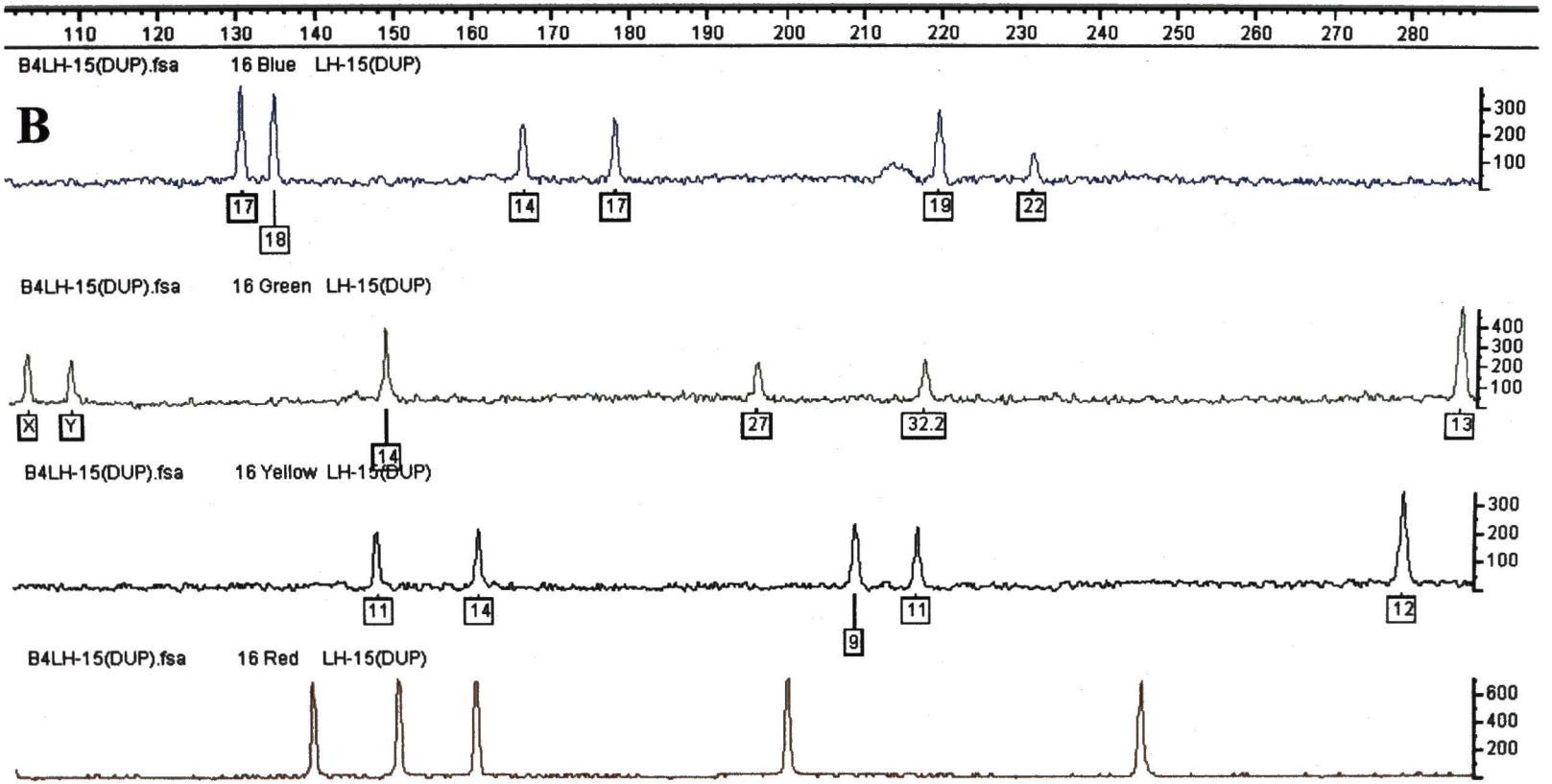

Figure 4B: Sample H. Second round of amplification to confirm profiles. B: Amplification 2, contains no unexpected alleles and produced a complete profile.

The reagent blank produced no alleles for either WGA or non-WGA amplifications, indicating that the 2pg/μL quantitation achieved by Q-PCR is either unreliable or the amount of DNA did not affect the amplifications. Figure 5 show the electropherogram for WGA amplification of the reagent blank. The source of the WGA positive control contained in the kit is lambda DNA and should produce no peaks with the human specific STR amplification. One WGA positive produce expected results, no peaks, while the other did not. The first WGA positive had no peaks and appeared as expected, Figure 6. The second WGA positive showed multiple peaks. Several of the peaks would indicate contamination from expected sources due to sample handling; however there are other peaks that would not. Table 6 shows the peaks that were called along with their RFU values. D3S1358 contains two alleles, 15 and 16, which do not correspond with the reference profile allele, 17, with RFU's values half of the corresponding allele. The 16 allele is in the stutter position; however the allele occurs at 38% of the major allele. This is well beyond the common limit of stutter, <15% (1). The D8S1179 locus contains two alleles, 14, corresponding to the reference profile, and the other in a stutter position, 13. However, this can not be stutter because the 13 allele is 88% of the 14 allele. A similar occurrence is seen in D5S818 locus, except the two alleles are separated by two repeat units and again the allele not associated with the reference profile has a higher RFU value. The sample was prepared from the same PCR amplification and analyzed on the ABI 310 again, Figure 7. The same peaks can be seen with similar circumstances.

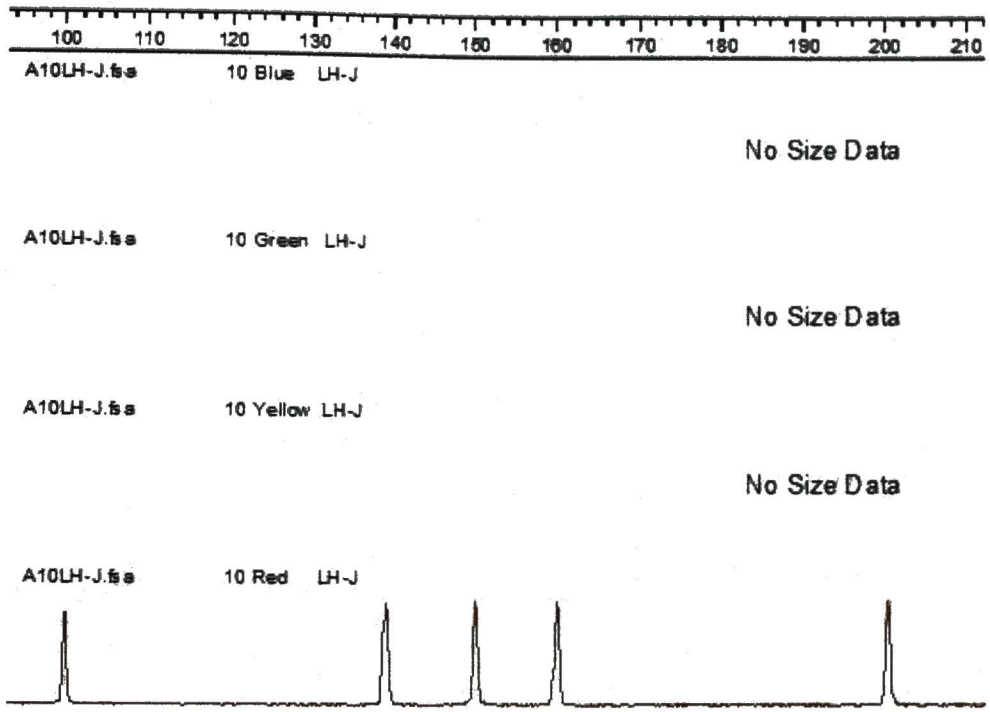

Figure 5: Reagent blank after whole genome amplification.

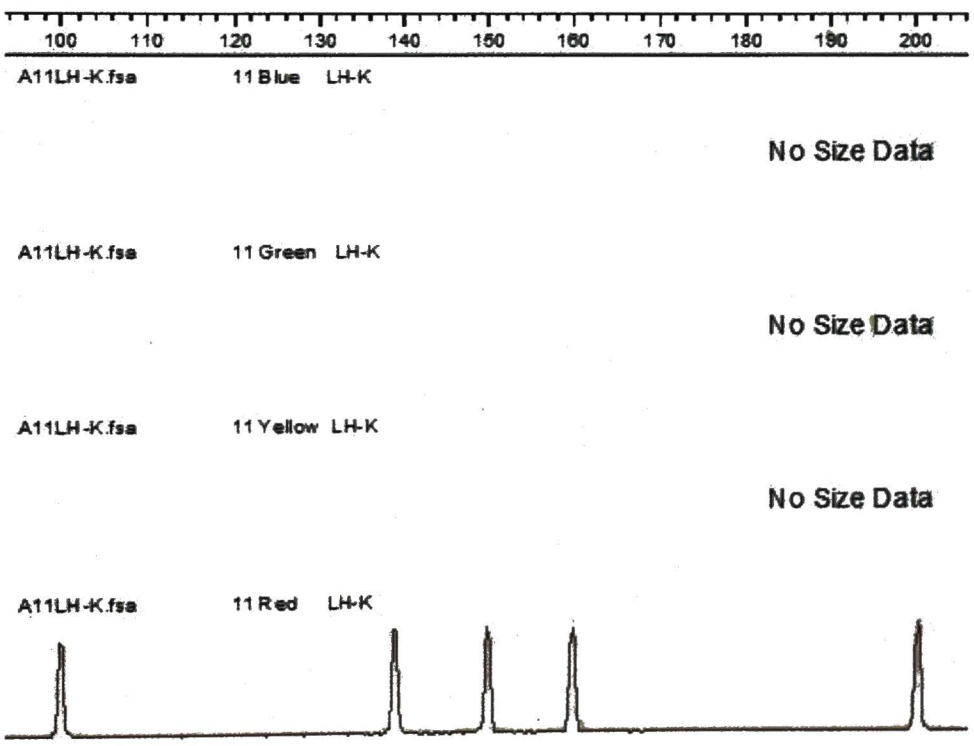

Figure 6: WGA Positive control of GenomiPhi reaction. Source was lambda DNA.

Table 6: Alleles present in positive control (Sample WGA Positive 2)

Locus	Allele	RFU
D3S1358	15	218
	16	233
	17	603
Amelogenin	X	1395
	Y	287
D8S1179	13	259
	14	292
D21S11	28	314
D5S818	9	432
	11	323

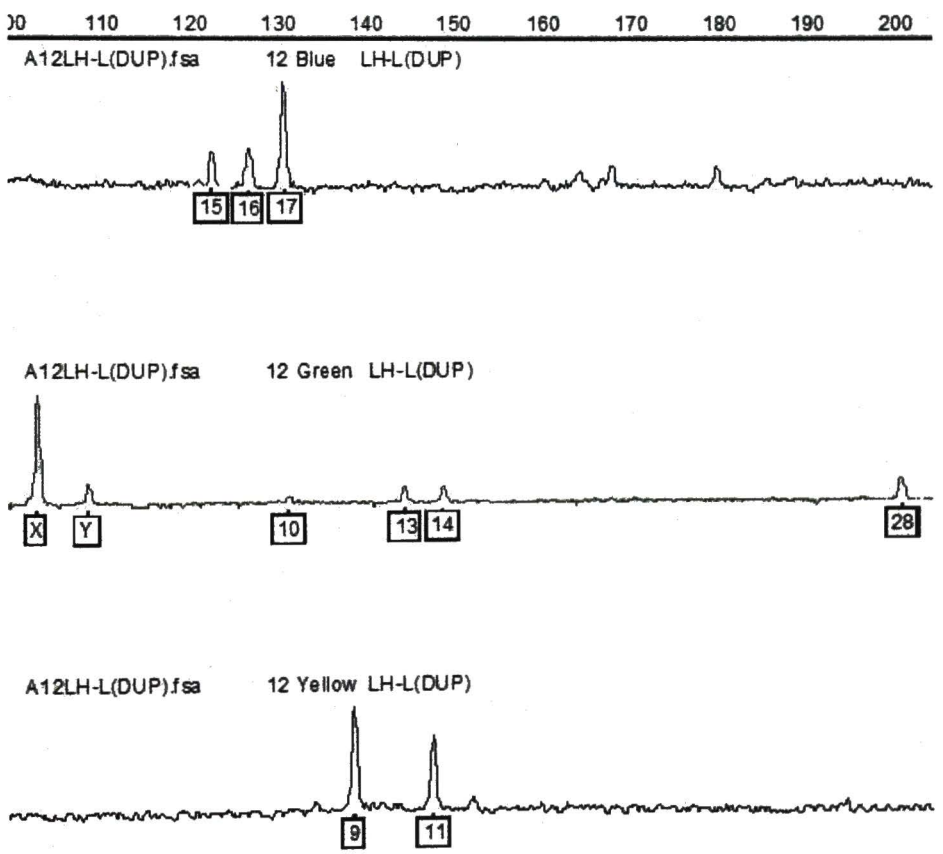

Figure 7: WGA Positive 2. Second round of amplification to confirm peaks. Source was lambda DNA.

CHAPTER IV

DISCUSSION

Whole genome amplification could provide the ability for the forensic community to lower the quantity of DNA that can be tested. The ability to obtain results from less than 100 picograms of DNA would increase the utility of genetic testing. With current technology, this means that WGA amplification would have to reliably amplify from less than 10 pg/μL of DNA. This study evaluated the commercially available WGA kit GenomiPhi to see if it was capable of this level of amplification. The kit states that it requires at least 1 ng of DNA to produce 4μg of product DNA.

Quantitation of unpurified product using the PicoGreen assay after WGA was the primary way to determine if amplification was successful before downstream analysis. The WGA positive control, which is lambda DNA and comes in the kit, at 10 ng of input DNA, should yield greater than 4 μg of DNA (200 ng/μL). The WGA positive controls produced the expected results for this study. Amersham Biosciences states that quantitation of blank samples will also show some amplification (19). What they do not state is how much amplification might be seen. Quantitation of reagent blank sample reported 197 ng/μl of DNA. This could be due to the fact that Q-PCR reported the reagent blank to contain 2pg/μL of DNA after extraction and the GenomiPhi kit is highly sensitive. However, no alleles were seen with STR analysis, Figure 5. This means that either the kit is not that sensitive or the Q-PCR values, which is below the standards, was

inaccurate. This defends the use of reagent blanks that contain minute amounts of DNA based on Q-PCR quantitation.

Results seen with the PicoGreen assay could be the fault of non-specific amplification during the WGA. The random primers used in the GenomiPhi kit are the cause behind all non-specific amplification. First is the possibility of primer dimmers being quantitated. Since PicoGreen is an intercalating agent it will show positive amplification, even if only primer dimmers are the only amplified product. Amersham Biosciences states that when genomic DNA is present the non-specific product from primer dimmers will not be produced due to the very inefficient amplification of the hexamers (19). Therefore this scenario of non-specific product only accounts for the quantitation of the reagent blank.

All samples showed greater than 200 ng/µl of DNA in WGA product. Since the reagent blank, according to the PicoGreen assay, produced almost as much signal as the actual samples, then the questions becomes what part of the actual samples was true genomic DNA and what part is background noise?

Before the standard curve of the PicoGreen assay is formulated, the RFU's are zeroed out by subtracting the zero standard from all samples. If this approach is taken with the actual samples using the reagent blank as the zero sample then the range of DNA produced from WGA is from 8 to 104 ng/µL for the samples taken through analysis. Then based on the dilutions that were made from WGA product, STR amplification would have occurred with 430 to 50 picograms of DNA, not the 1.25 ng which dilutions were calculated for. If this was the case, sample E would be amplifying 50 picograms of

DNA, and yielding a full profile, while the I sample would be amplifying 100 picograms of DNA and producing less than half the expected alleles. Since most likely this is not the case, quantitation of WGA product prior to STR analysis needs some refining.

Real time PCR was performed on a set of test WGA samples, before the other samples were amplified with GenomiPhi and analyzed. Starting DNA was control DNA provided in Applied Biosystems Quantifiler kit. Seven hundred thirty nine picograms to 8 pg of DNA were amplified with the GenomiPhi kit. The product was diluted 20 fold by combining 5 μL of product with 95 μL of TE^{-4}. Q-PCR and PicoGreen results are shown in Table 7.

Since Amersham states that 1 ng of input DNA is required to achieve 200 ng/μL, it was expected that the addition of 739 pg of DNA would have yielded the largest quantity of DNA. After Q-PCR was preformed with the Quantifiler kit, samples were quantitated with the PicoGreen assay. Since the PicoGreen values reported quantities expected from WGA product, the Quantifiler results were considered unreliable. There may be possible locus bias in the WGA amplification for the human telomerase reverse transcriptase gene used by the Quantifiler kit (2). Especially since one sample, 406 pg of input DNA did produce expected results. Further studies should be performed to evaluate the functionality of Q-PCR on WGA products and determine if modifications can produce reliable results.

Another possibility for the quantities of DNA seen in all samples is contaminating DNA from the samples themselves. Mitochondrial genomes would have also been extracted with genomic DNA and be available for WGA in the samples. The

mitochondrial genome is approximately 16.5 kilobases in length. If the Phi29 polymerase were to initiate replication it would be able to encompass the circular genome four times with every primer annealing site. Bacterial DNA could also have been present and produce double stranded product that would give results with PicoGreen assay but not produce profiles with STR amplification. This may account for the high DNA yields, but lack of complete profiles.

Table 7: Quantifiler results of WGA product

Input DNA	Quantifiler	PicoGreen
0.739	4.900	354.19
0.502	5.180	368.27
0.406	211	351.43
0.132	7.280	358.06
0.034	0.612	333.22
0.043	0.648	355.02
0.030	0.108	355.57
0.008	Undetermined	372.68
WGA Positive	Undetermined	385.65

Q-PCR values of WGA product compared to PicoGreen. Input is total nanograms of DNA. Quantifiler and PicoGreen values are in ng/μL.

Whole genome amplification on the degraded sample yielded the following values for three separate amplifications: 14.55, -5.03, and 0.60 ng/μL of DNA. The -5.03 is a result of subtracting the zero standard from all samples for the PicoGreen assay. A possible cause of the DNA quantities observed in the degraded sample is the presence of EDTA in the solution, which was used to stop the degradation reaction. EDTA binds magnesium which is a cofactor of DNA polymerases. The presence of too much EDTA in the sample would therefore have decreased the functionality of the Phi29 polymerase. Increasing the magnesium concentration of the WGA reaction may correct for this.

However, with use of the GenomiPhi kit, the concentration of the magnesium in the buffer is proprietary, so determining appropriate magnesium levels may be difficult.

STR analysis of non-WGA samples produced results in at lest one amplification for all samples except sample I. Figure 4B shows sample H, the lowest sample quantity, approximately 360 pg of DNA, which yielded a complete profile. Sample I would have shown a full profile for amplification 1, Figure 8, if the RFU threshold had been set at 100. The peaks that are not labeled at the 150 threshold level can clearly be seen. The second amplification would produce all alleles except the Y allele if the RFU threshold was lowered. Table 10 in the Appendix lists the RFU values of the missing alleles.

Signs of possible contamination appeared in two samples, H non-WGA amplification 1 and WGA Positive 2 WGA amplification 2. Table 8 list the alleles common between the two samples and their RFU values.

Table 8: Common alleles between contaminated samples

		RFU	
		H	WGA Positive 2
D3S1358	15	1272	188
	16	1116	202
	17	3931	501
AMEL	X	5870	1328
	Y	1759	311
D8S1179	13	1399	271
	14	1797	241
D21S11	28	1777	327
D5S818	9	2924	386
	11	2232	289

Sample H is non-WGA and WGA Positive 2 is lambda DNA control from GenomiPhi kit

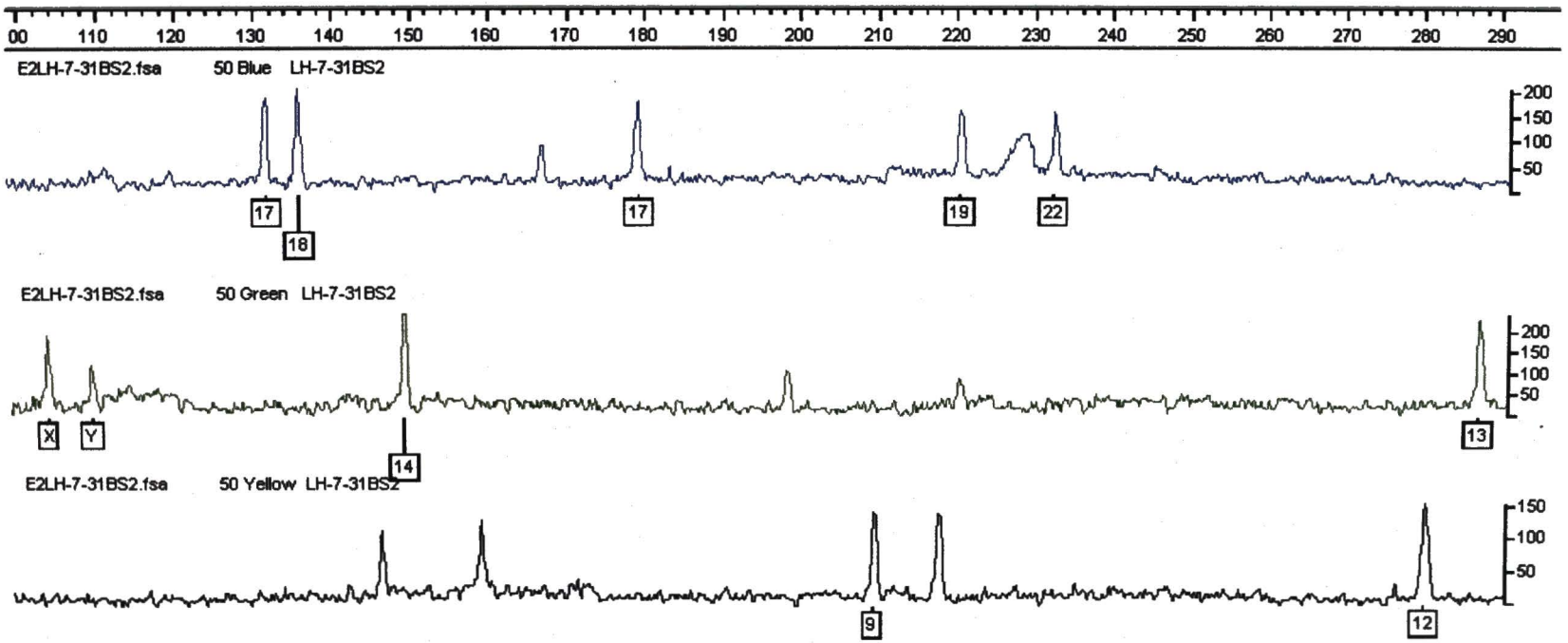

Figure 8: Sample I. Approximately 150 pg of DNA being amplified by PCR. Complete profile possible if RFU threshold set to 100.

WGA positive 2 should have produced no peaks since the source of the DNA was the lambda control from the GenomiPhi kit. It appears that the majority of the contamination is not from the male volunteer. Several alleles are common to his profile; D3S1358-17, Amelogenin, D8S1179-14, D5S818-11, but the imbalance in the amelogenin locus indicates the primary contributor may be female. There were several other alleles, which would indicate multiple contributors in the H sample, Figure 4A, which did not appear in WGA positive 2 sample. This could be due to the fact that the RFU values in WGA Positive 2 are about 15% of the H sample and at that reduced level they would fall below the RFU threshold. Data reported is for re-injections of the samples. This confirms the presence of the alleles and that contamination had to occur while sample were being prepared for PCR, since the duplicate amplifications did not show similar profiles.

Locus dropout was seen in several samples, especially in the WGA samples. Figure 9 shows locus dropout of vWA, FGA, D8S1179, and D13S317 in sample H WGA. There is also allelic dropout of 18 at D3S1358, X at amelogenin, and 27 at D21S11. This is a very good example for allelic dropout and locus dropout not only for the combination of the two in one sample, but that the alleles that are present have moderately high RFU's. Also of interest in this sample is that the X allele drops out. Typically this will be the last allele to disappear due to its small size.

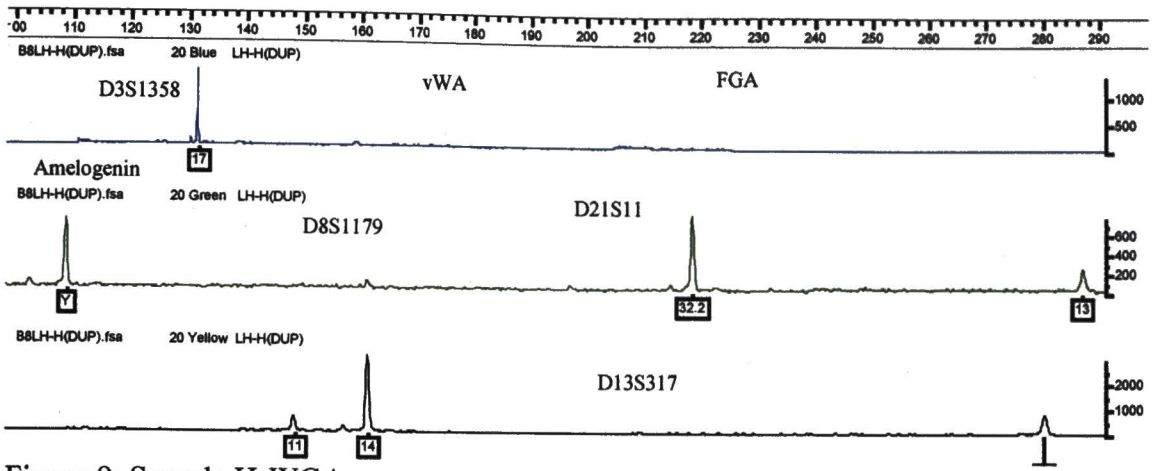

Figure 9: Sample H-WGA.

Allelic dropout is a concern when dealing with low copy number samples. The samples used in this study were not true LCN, as no samples were amplified for STRs with less than 100 pg, and still the effects of this problem were seen. Sample F of the WGA products showed a good example of allelic dropout, Figure 10. The 27 allele of D21S11 is clearly missing from around 195 bases. Locus D13S317 seen in the yellow dye is also missing the 11 allele. There is the start of a small peak appearing where the 11 would exist, but nothing can be confirmed. All the other alleles seen in Figure 11 are heterozygous, like the 27 and 11, and have moderate RFU values.

During the preparation of the degraded DNA sample an agarose yield gel was run to compare a degraded to a non-degraded sample. Visualization with ethidium bromide showed what appeared to be no degradation. Figure 11 shows the resulting profile obtained from the degraded DNA, without WGA, indicating that degradation did in fact occur.

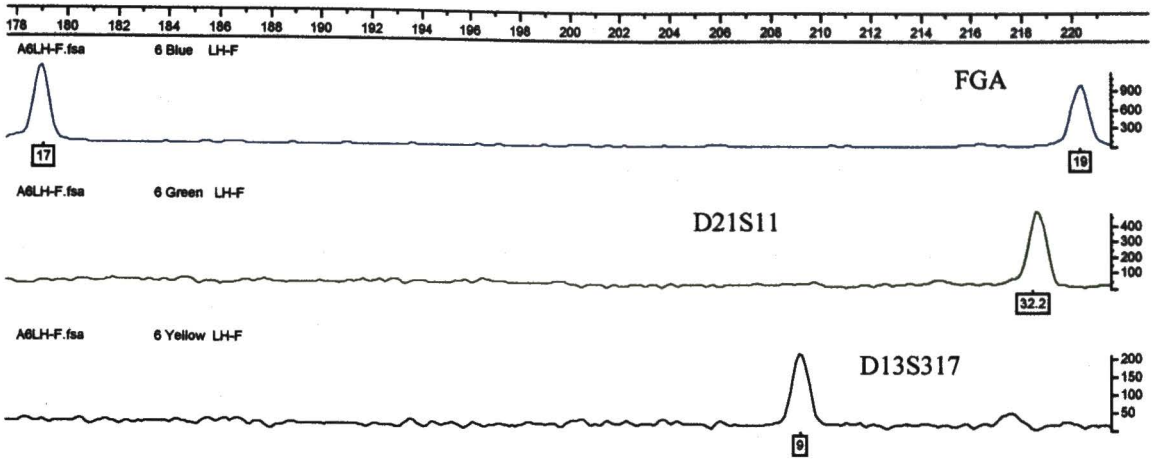

Figure 10: Sample F-WGA. 27 allele, around 195 base pairs, of D21S11 is missing from the green dye. 11 allele, around 218 base pairs, is missing from D13S317 in the yellow dye. All other alleles seen are heterozygotes.

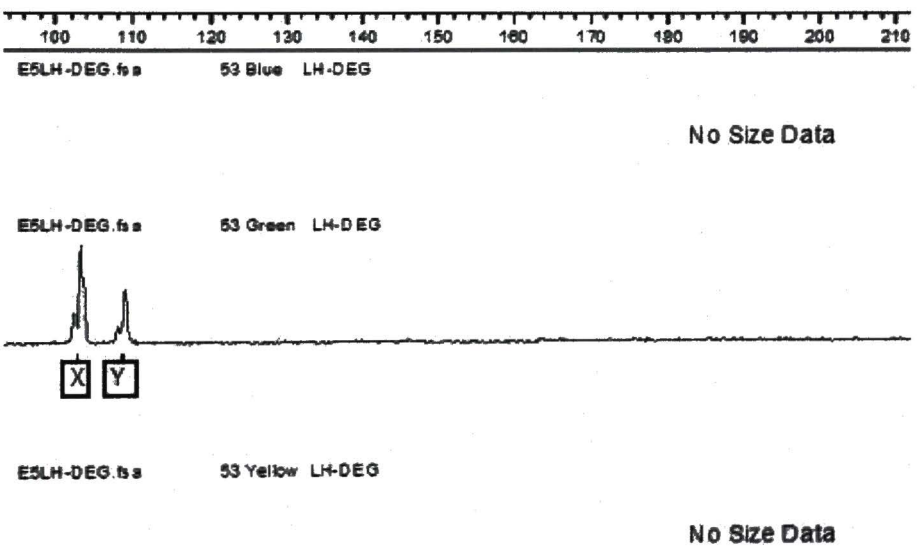

Figure 11: Degraded DNA sample of non-WGA.

The only locus that amplified in the non-WGA was amelogenin. WGA amplification on this sample did produce two new peaks. As seen in Figure 12, an 11 allele was produced at D5S818 and 14 at D8S1179. These alleles are expected from our

volunteer. Another allele did appear at the D5S818 locus, but the RFU of the 14 allele was 126. The D13S317 locus appeared to be under-amplified in the whole genome amplifications. It was the first loci to lose an allele in one of the amplifications of sample D. The 11 allele was only seen down through sample E and the 9 allele down through the F sample. The 9 allele did appear once more in sample I.

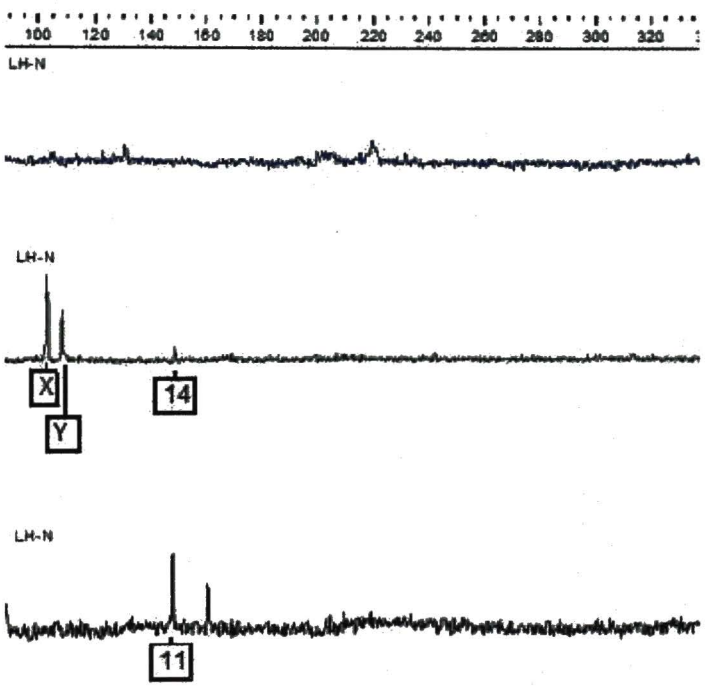

Figure 12: Degraded DNA after whole genome amplification.

Allelic peak imbalance can be an important factor when analyzing samples. Considerable peak imbalance, greater than 30% difference, is typically only seen in mixtures. When there is considerable difference between heterozygous peaks this could mean that a mixture is present. Greater heterozygous peak imbalance was seen with the

GenomiPhi samples than the non-WGA samples. Table 9 reports the average percent difference of heterozygous peaks for non-WGA and WGA samples.

All the non-WGA samples, except the degraded sample, have acceptable peak imbalance levels. The only alleles present in the degraded sample were the X and Y of amelogenin. Most of the WGA samples are above the cutoff limit of 30%. The degraded DNA sample's peak imbalance was reduced with WGA. None of the expected heterozygous loci contained both alleles for sample I-WGA.

Table 9: Average percent difference of heterozygous peaks

	Non-WGA	WGA
A	10.90	14.91
B	13.39	19.08
C	16.33	34.84
D	13.47	34.59
E	19.77	23.55
F	11.42	31.82
G	10.88	38.21
H	14.67	74.83
I	26.40	- - -
Non-degraded	11.58	14.66
Degraded	50.93	36.36

Sample I WGA did not contain both alleles for any heterozygous loci.

Overall the whole genome amplification reaction did not increase the limit of amplified DNA. Complete profiles were obtained from less DNA without WGA than when using it (Table 5). One goal was to determine the amount of DNA that the GenomiPhi kit would produce if less than optimal amount of starting material was used.

The results showed that even when little to no DNA was used, as with sample I and the reagent blank, the PicoGreen assay would quantitate samples to about the same quantity of DNA.

The manufacture states that when using the PicoGreen assay it is not necessary to clean up the WGA product, the question is whether or not some type of post amplification clean up would produce better results? Amersham recommends alcohol precipitation and spin column chromatography if clean up is desired. These methods will remove the unincorporated nucleotides, primers, and most small DNA fragments. In reagent blanks the removal of the primers and primer dimmers could lower the false quantitative values. The removal of very small fragments of DNA, produced from the early stages of a displaced strand could also aid in correcting the PicoGreen results for the rest of the samples. However if the values obtained are based on amplification of nuclear, mitochondrial and bacterial genomes in the samples then the quantities achieved are very close to accurate and these methods will not greatly decrease the quantity of DNA.

Other methods are possible that could accurately quantitate the human DNA of the WGA product. Q-PCR was attempted on a set of test samples, but results were inconsistent. This could be due to a couple of reasons. One is that there is uneven amplification of the locus utilized by Applied Biosystems Quantifier kit. A second reason could be that the GenomiPhi primers left over from the WGA reaction are being amplified as opposed to the Quantifiler primers. However, if this was the case, then interference with the internal positive control should have also been observed and it was

not. Also, the temperature used for annealing and extension during Q-PCR, 60°C, is higher than the 30°C used during the GenomiPhi reaction. Other real time systems have been used to quantitate GenomiPhi product (20).

Fewer complete profiles were seen with the GenomiPhi kit. One possibility for this is that the reaction is not reliable with very small amounts of DNA, less than 250 picograms. A second possibility for this is that the multiplex of the Profiler Plus kit was unbalanced. Multiplex PCR reactions are a very balanced equation of primers, template DNA, polymerase, dNTP's, salts, cofactors, and adjuvants. When the WGA samples were added to the Profiler reaction, some of these components were most likely carried over into PCR. Several problems may arise from the introduction of these items. Any of these components may disrupt the balance of the PCR reaction and lower the efficiency of amplification. If any random hexamer primers were carried over they could compete with the STR primers for amplification by Taq polymerase. Amersham says that the hexamer primers should not effect the multiplex reaction since the Tm of the primers is so low, unless the reaction contains annealing temperatures around 30°C. The annealing temperature of our PCR multiplex was 59°C. A post WGA clean up would alleviate either of these problems by removing unincorporated primers, salts, cofactors and adjuvants.

Whole genome amplification has been shown to produce results from as little as one cell worth of DNA (15). This was accomplished using primer extension preamplification. This study has shown that when using the GenomiPhi kit, reproducible and reliable results can only be received from samples as low as 234 pg/μL. When

amplifying STRs, in downstream applications, this concentration of DNA would provide the optimal amount of about 1 nanogram of DNA to amplify in PCR. While the GenomiPhi kit does work as described, this study has determined that there is little utility for it, currently, in the forensic community. Future studies need to investigate modifications to the GenomiPhi guidelines or non-commercially available kits that may be optimized by each laboratory to fulfill their needs.

APPENDIX

Table 10: Profiles from non-WGA samples

		Samples																							
		A		B		C		D		E		F		G		H		I		Reaget Blank		Degraded		Non-degraded	
Locus	Alleles	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
D3S1358	17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	---	---	---	---	+	+
	18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	---	+	+	+	---	---	---	---	+	+
vWA	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	109	+	---	---	---	---	+	+
	17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	---	+	+	+	---	---	---	---	+	+
FGA	19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	101	---	---	---	---	+	+
	22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	---	---	---	---	+	+
Amelogenin	X	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	---	---	+	+	+	+
	Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	---	---	---	+	+	+	+
D8S1179	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	---	---	---	---	+	+
D21S11	27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	---	+	127	135	---	---	---	---	+	+
	32.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	---	+	113	+	---	---	---	---	+	+
D18S51	13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	---	---	---	---	+	+
D5S818	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	122	+	---	---	---	---	+	+
	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	---	+	140	+	---	---	---	---	+	+
D13S317	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	---	---	---	---	+	+
	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	144	117	---	---	---	---	+	+
D7S820	12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	---	---	---	---	+	+

Pluses (+) indicate that the allele was present with a 150 RFU threshold. Numbers indicates RFU value for alleles called in the 100 to 150 range. Dashes mean that the allele was not called. Sample 15-1 also showed several other peaks with similar RFU values to the alleles.

Table 11: Profiles from WGA samples

		Samples																									
		A		B		C		D		E		F		G		H		I		Reagent Blank		Positive 1		Positive 2		Degraded		Non-degraded	
Locus	Alleles	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
D3S1358	17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	--	--	--	--	--	--	--	+	+	+	**126**	**107**
	18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	--	--	--	--	--	--	--	--	--	--	+	+	--	--
vWA	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	--	--	+	+	--	--	--	--	--	--	+	+	--	--
	17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	--	--	--	--	--	--	--	--	--	--	+	+	--	--
FGA	19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	--	--	+	**118**	--	--	--	--	--	--	+	+	**135**	--
	22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	--	--	--	--	--	--	--	--	--	--	+	+	--	--
Amelogenin	X	+	+	+	+	+	+	+	+	+	+	+	**120**	+	+	--	--	+	+	--	--	--	--	--	+	+	+	+	+
	Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	--	--	--	--	--	--	--	+	+	+	+	+
D8S1179	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	**110**	**100**	--	--	--	--	--	--	--	+	+	+	+	--
D21S11	27	+	+	+	+	+	+	+	+	+	+	--	+	+	+	+	--	+	+	--	--	--	--	--	--	+	+	--	--
	32.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	--	--	--	--	--	--	--	--	+	+	--	--
D18S51	13	+	+	+	+	+	+	+	+	+	+	+	+	--	**126**	+	+	--	--	--	--	--	--	--	--	+	+	--	--
D5S818	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	--	--	--	--	--	+	+	+	+	**121**
	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	**107**	--	--	--	--	--	--	--	+	+	**126**	+
D13S317	9	+	+	+	+	+	+	**105**	+	+	+	+	+	--	--	--	--	+	--	--	--	--	--	--	--	+	+	--	--
	11	+	+	+	+	+	+	+	+	+	+	--	+	--	--	--	--	--	--	--	--	--	--	--	--	+	+	--	--
D7S820	12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	**117**	--	--	--	--	--	--	+	+	--	--

Pluses (+) indicate that the allele was present with a 150 RFU threshold. Numbers indicates RFU value for alleles called in the 100 to 150 range. Dashes mean that the allele was not called. Sample Postive 2-1 also showed several other peaks with similar RFU values to the alleles. Postive control are lambda DNA from GenomiPhi kit

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