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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

DEVELOPMENT OF A MICROWAVE-BASED DNA EXTRACTION METHOD TO INCREASE THE SUCCESS OF DIRECT AND RAPID PCR TECHNIQUE

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

Fabiana Taglia

2022

To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Fabiana Taglia, and entitled Development of a Microwave-based DNA Extraction Method to Increase the Success of Direct and Rapid PCR Technique, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

Steven B. Lee

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Jeffrey Wells

Bruce McCord, Major Professor

Date of Defense: March 30, 2022

The dissertation of Fabiana Taglia is approved.

Dean Michael R. Heithaus
College of Arts, Sciences and Education

Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2022

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DEDICATION

I dedicate this dissertation to my family, my parents, grandparents, and my sister for always believing in me, supporting my journey, despite the distance. I have always been told how brave I was for leaving my country alone without any certainty of what I would do once here. This courage comes from my family because they allowed me to pursue my dreams always surrounded by their constant love.

Thanks to my best friends in Italy, always ready to listen and cheer me, to the friends here that helped me in any way they could. to take care of me and my son when I needed them the most. Thanks to Georgiana, Chiara, Mirna, Roberta, Nicole, Ling, Joana, and everyone in Dr. McCord's Labs, you know the contribution you had.

They said the real friends are those you can count on your hand, but that's not true for me, I have been blessed with so many caring people no one can imagine.

The genuine love you all showed me, pushed me always to move forward without stopping

Finally, I dedicate this work to my smart kid, my future, my legacy.

Dear Milo, thank you for challenging me every day, making my work less easy but helping me growing stronger.

You are the best that could ever happen to me.

ABSTRACT OF THE DISSERTATION

DEVELOPMENT OF A MICROWAVE-BASED DNA EXTRACTION METHOD TO INCREASE THE SUCCESS OF DIRECT AND RAPID PCR TECHNIQUE

by

Fabiana Taglia

Florida International University, 2022

Miami, Florida

Professor Bruce McCord, Major Professor

The goal of this project was to develop a fast, microwave-based extraction technique that could be employed for direct and rapid DNA analysis. The hypothesis was that the use of a microwave could increase the yield of DNA by opening the cell membrane, rendering the DNA available without the use of any other chemical treatment, and improving results from very low quantity samples.

At present rapid human DNA analysis is mainly restricted to genotyping saliva and sometimes blood samples. We hypothesized that microwave processing could expand the types of samples assessable to these procedures and increase sensitivity.

There were two different microwave ovens employed: a commercial one and a computer-driven microwave. The samples came from three different body fluids: saliva, semen, and blood. Control samples were prepared using the same samples that were not subjected to microwave extraction. The quality and quantity of DNA profiles obtained from these experiments were assessed using a novel PCR-based analysis, a homemade multiplex consisting of 9 simultaneously amplified mini STR loci. A direct PCR method was developed to analyze samples rapidly with minimal pretreatment and no extraction.

Once optimized, this procedure was developed for touch DNA samples and mixtures. The optimized microwave protocols were also assessed using a completely automated commercial rapid DNA instrument. We hypothesized that preprocessing by microwave digestion could widen the application of these commercial systems, which are generally designed to analyze oral swabs.

The evaluation of the methodologies was performed using statistical tests (ANOVA or T-test) on allele call rates, peak heights, and allelic concordance for all loci. Overall, the use of a microwave coupled with rapid direct PCR, represents a valuable addition to an analyst's toolkit, increasing the DNA yield as well as the speed of the experiments. Moreover, it is less expensive and less toxic than other pretreatments making it an excellent procedure for onsite analysis.

This project was supported by the Irregular Warfare Technical Support Directorate (IWTSD, formerly CTTSO) of the US Department of Defense. The research started in October 2019 and was completed in May 2021.

TABLE OF CONTENTS

CHAPTER..……PAGE

CHAPTER 1. Overview……1
1.2 Significance of the study and impact……2
1.3 Dissertation structure……3

CHAPTER 2. Background and literature review……...5
2.1 History of DNA analysis in forensic investigations…….5
2.2 Biological Evidence……15
2.2.1 Saliva……...…….....15
2.2.2 Blood……....16
2.2.3 Semen……...18
2.3 DNA Extraction……..19
2.4 Microwave Extraction…….....24
2.5 Literature review…….26
2.6 DNA Amplification.....31
2.6.1 Rapid PCR.....32
2.6.2 Direct PCR……...37
2.6.3 Rapid-Direct PCR……....38

CHAPTER 3. Experimental Methods - Microwave oven Calibration……40
3.1 Introduction……..40
3.2 Neon Bulb and sample retention tests……..41
3.2.1 Materials and methods……..41
3.2.2 Results-Discussion……....43
3.3 Temperature tests……45
3.3.1 Materials and methods……..45
3.3.2 Results and Discussion……45

CHAPTER 4. Conductivity……48
4.1 Introduction……48
4.2 Materials and methods……...48
4.3 Results and Discussion……..49

CHAPTER 5. Quantification……..50
5.1 Introduction……..50
5.2 Materials and methods…….51
5.2.1 Sample collection and microwave extraction……...51
5.2.2 Real-Time PCR and Fluorometric quantitation……51
5.3 Results and Discussion……53
5.4 Optimization of computer driven microwave……..56

CHAPTER 6. Amplification and Genotyping…….59
6.1 Introduction……59
6.2 Materials and methods……...59
6.2.1 Direct Polymerase……...59
6.2.2 PCR amplification……...62
6.2.3 Genotyping…….….67
6.3 Results and Discussion ……70
6.3.1 Saliva……..70
6.3.2 Blood……..83
6.3.3 Semen……86
6.3.4 Mixtures……..90

CHAPTER 7. Collaborative Research with NFSTC, MDPD, PBSO…….....97

CHAPTER 8. Conclusion and Future work……...……99

References…….….101

VITA……..111

LIST OF TABLES

TABLE…….....PAGE

Table 1. List of STRs, primers' sequences and relative fluorescence assigned.....63

Table 2. Primer's list, concentrations, and amount used to create the primer mix.
The total volume of the primer mix created was 38µl,
from this total volume we used 3.6µl for the PCR mix.....65

Table 3. Master mix direct/fast PCR amount per sample, final volume 10µl.....65

Table 4. PCR cycling conditions optimization performed using Philisa Streck Thermal cycler.
Three different protocols were used: A, B, and C.....66

Table 5. Results obtained from the amplification and genotyping of positive
control 2800M 1ng/µl using three different protocols: A, B, and C (Table 4).
Peak heights are shown in terms of the relative unit of fluorescence (RFU).....66

Table 6. Results obtained from the amplification and genotyping of positive
control 2800M 1ng/µl, 0.5ng/µl, 0.25ng/µl and 0.125ng/µl using B protocol.
Peak heights are shown in terms of the relative unit of fluorescence
(RFU).....67

Table 7. Results obtained from the blood sample diluted 1%,
peak heights average, and percent of increase are shown.....85

Table 8. Results obtained from the semen sample diluted 0.5%,
peak heights average, percent of increase are shown.....89

Table 9. Genotype of the mixture contributors; female saliva and male
semen.....90

Table 10. Summary of MDPD and PBSO laboratory results.....98

LIST OF FIGURES

FIGURE…….....PAGE

Fig.1. Schematic representation of the two types of polymorphisms in the human genome, sequence polymorphisms (SNPs) and length polymorphisms (VNTRs and STRs).....6

Fig 2. RFLP results in OJ Simpson casework. The autoradiography represents blood evidence collected and compared with the profile of the two victims and the suspect. Image from https://mordundtotschlag.wordpress.com/2013/02/19/der-prozess-von-o-j-simpson/.....7

Fig.3. PCR amplification mechanism.
The three steps are shown: denaturation, annealing and extension. In the first step of denaturation the sample is denaturated using high temperature usually around 95ºC for few seconds. Once the two strands are denaturated the primers can pair with the DNA at a certain annealing temperature. After the binding of the primers, the polymerase can start the extension step and polymerize the new DNAfragments
that cycle after cycle will produce thousands of copies of the original DNA.
Image from https://commons.wikimedia.org/wiki/File:Polymerase_chain_reaction-en.svg.....9

Fig 4. AMP-FLP results on D1S80 using silver staining technique (Schanfield 2000).The lane C is the control, the L is the ladder, and the numbers represent the samples and their pattern.....10

Fig 5. A, B, and C. Electropherograms showing three different DNA samples: A. degraded sample, B. low copy sample, and C. mixture sample. Saliva samples were prepared and treated from individual donors, collected on a buccal swab, treated, and amplified with the Investigator 24plex QS STR kit, Qiagen (QIAGEN inc, Valencia, CA).....11

Fig 6. Mitochondrial DNA structure (left) and examples of polymorphisms on the Y chromosome (right) (Butler 2005).....13

Fig. 7. Schematic of a RSID blood test. The sample is introduced in the sample (S) window, C is the control lane, T is the test lane. Three are the possible outcomes; 7a

positive result, 7b negative result or 7c inconclusive result. Image created by the author.....17

Fig. 8. Organic and Chelex extraction mechanism
In the organic extraction (top) the sample is treated with a detergent, sodium dodecyl sulfate (SDS), and Proteinase K to lyse the cells, after an incubation step, Phenol-Chlorophorm is added. The following centrifugation step allows the separation of the sample in phases. The nucleic acid if interest will be found in the aqueous layer. Once removed it can be transferred and retained into a clean tube. In Chelex extraction (bottom) the sample is boiled with water and Chelex resin. The resin binds to the Mg^{2+}, leaving the DNA in the aqueous solution ready to be transferred and analyzed (McKiernan 2017).....20

Fig. 9. Schematic of Solid Phase Extraction (SPE).
The sample is treated with a lysis buffer and paramagnetic beads are introduced. In the presence of high salts concentration, the DNA will bind to the beads. A magnetic stand is used to keep the DNA bound. Several washing steps are included to clean the sample from cellular debris. Once done with the cleaning step, the DNA can be eluted and retained for further analysis (McKiernan 2017).....22

Fig.10. Electromagnetic spectrum. Reproduced from NASA http://science.nasa.gov/ems/06_microwaves.....24

Fig.11. Microwaves radiation path in a commercial microwave oven.
In the figure is shown the possible radiation path and the tubes with biological samples that can be introduced in the microwave and exposed to the radiation. Edited from Malheiro (2011).....25

Fig.12. Microwave exposure effect on plant cells. The exposure to 15-30 min of microwaves, in the example provided, allows the large recovery of analytes when compared to common methods of extraction (Mukherjee 2019).....26

Fig.13. Microwave extraction method applied to several bacterial strains. The results demonstrated that DNA yields increased after microwave treatment compared to the standard extraction technique (Bollet 1991).....28

Fig.14. Comparison of DNA yields and purity using Microwave irradiation vs Boiling and Enzymatic Extraction. For both bacterial strains, microwave extraction

produced an increase in both yield and purity (Omar 2014).....29

Fig.15. The transmission electron microscope (TEM) photography of Bacillus cereus in the control group (× 2,500) (a) and in the treated group (× 2,500) (b), respectively. The effect is clear, the microwave treatment inactivated the spore (Cao 2018).....30

Fig.16. Electrophoretic gel showing a comparison between common extraction (CE) and Microwave extraction (ME) for a variety of substrates. The results for ME extraction are comparable in quality and quantity to those observed for CE extraction (Giberson 1995, Thomas 2018).....31

Fig. 17. Comparisons between standard thermal cycler (A, B) and rapid instrument (C, D). A β-globin protein of 536bp was amplified according to different amplification protocols. The results in A and B using standard protocol (4 and 2 hours respectively) showed the presence of non-specific products on the gel, while faster protocols C, and D performed in 40 and 15minutes showed clear bands on the gel. (Wittwer 1994).....33

Fig 18. A, B, C, and D. Optimization Rapid amplification protocol. The effect of several parameters' optimization is shown. In 18A the limit annealing time, less than 1 sec provided higher specificity. The effect of elongation time in 18B demonstrated that short elongation time can be used without affecting the results. In 18C and 18D the effect of EtBr on the amplification process is shown. The results suggest that the use of EtBr does not affect the amplification (18C) and can be used to monitor of PCR performance (18D) (Wittwer 1994).....34

Fig.19. Electropherogram showing 100 ng of K562 control DNA standard. Results from 7 STR loci amplified using ultra-rapid PCR amplification (Gibson-Daw 2018).....39

Fig.20. Commercial Microwave oven used for preliminary calibration tests (left), and sample racks registration inside the oven.....40

Fig.21. Neon bulbs set-up. First the electrodes were cut to fit the tube length, then they were carefully placed in the tube avoiding any contact between the filaments.....41

Fig.22. Arrangement of 1.5 ml tubes in the sample racks. The tubes were filled with 100µl of water and exposed to several cycles of microwaves.....42

Fig.23. Picture taken while neon bulbs were exposed to the microwaves. It is possible to visualize the different lighting pattern create by the radiation.....43

Fig.24. Location selected. The preliminary results in Microwave oven calibration allowed the selection of 4 preferable locations for the samples.....43

Fig.25. Example of different status of condensation observed after microwave oven exposure. a=Lower level of condensation, b= Medium level of condensation, c= High level of condensation.....44

Fig.26. Relation between temperature and volume in the selected location, standard error bars are shown.....46

Fig.27. Relation between temperature and different microwave heating cycles, standard error bars are shown.....47

Fig.28. Conductivity meter Horiba B-173 Twin Cond.....48

Fig.29. Conductivity test results. Three buffers, Tween-20, TE and Water, were used to dilute a saliva sample using the following dilution scheme: saliva not diluted, diluted 75%, 50%, 25%, 10% and only buffer. Once diluted, conductivity measures were taken. Each sample was measured 3 times. Errors bars are shown.....49

Fig.30. Quantification test on same donor saliva. A saliva sample, from the same donor, was collected, diluted 75% and quantified (3 replicates) each day in a period of 5 days. Standard deviation bars are shown.....54

Fig.31. Results of quantification tests on diluted saliva with water, TE, and Tween-20.

A saliva sample was diluted using the three different buffers at several percentage of dilution: saliva not diluted, diluted 75%, 50%, 25%, 10% and only buffer. The samples were then quantified in replicates of 3 for each buffer and dilution fraction.....55

Fig.32. Optimization computer driven microwave. Test 1. Saliva samples were microwaved in triplicates for 0s, 10s, 20s, 30s, 40s and 50s at the power of 700W. Once microwaved they were quantified. Error bars are shown.....57

Fig.33 Optimization computer driven microwave. Test 2. Saliva samples were microwaved in triplicate for 40 sec at different energy levels: no microwave, 300W, 400W, 500W, 600W and 700W.....57

Fig.34. Optimization computer driven microwave.
Test 3. Saliva samples were microwaved in triplicate for 40 sec at lower energy levels: no microwave, 100W, 150W, 200W, and 300W.....58

Fig. 35. Fast Thermal cycler Philisa® from Streck Biosciences.
Details of the heat block and the design of the tubes are shown.....61

Fig.36. Allelic Ladder capillary electrophoresis, raw data. The starting point of TH01 is indicated by the arrow.
The raw data shows the primer peak overlapping with the peak of TH01.
Instrument used, ABI 3130XL.....64

Fig.37. Illustration showing an example of the mechanism of detection using gel electrophoresis. Butler, 2004.....69

Fig.38. a, b, c, d, and e. Electropherogram of donor 1,
female saliva sample diluted 1%.
In the boxes, the peak heights in terms of RFU are shown.....71-73

Fig.39. a, b, c, d, and e. Electropherogram of a female saliva sample, donor 1, diluted 1%. In the boxes, the peak heights in terms of RFU are shown.....74-76

Fig.40. a, b, c, d, and e. Electropherogram of a female saliva sample, donor 2, replicate 1, diluted 1%.
In the boxes, the peak heights in terms of RFU are shown.....77-79

Fig.41. f, g, h, i, and j. Electropherogram of a female saliva sample,
donor 2, replicate 2 diluted 1%.
In the boxes, the peak heights in terms of RFU are shown.....80-82

Fig.42. a, b, c, d and e. Electropherogram of donor 2,
female blood sample, diluted 1%.
In the boxes the peak heights in terms of RFU are shown.....83-85

Fig.43. a, b, c, d, and e. Electropherogram of the male semen sample,
donor 3, diluted 0.5%.
In the boxes the peak heights in terms of RFU are show.....86-88

Fig.44 a, b, c, d, and e. Electropherogram of mixture 1:9 female saliva,
and male semen.
In the boxes the peak heights in terms of RFU are show.....91-93

Fig.45. a, b, c, d, and e. Electropherogram of mixture 1:1, female saliva,
and male semen.
In the boxes the peak heights in terms of RFU are shown.....94-96

ABBREVIATIONS AND ACRONYMS

ºC Degree	Celsius
ABI	Applied Biosystem by Life Technologies
Bp	Base Pair
BSA	Bovine Serum Albumin
CE	Capillary Electrophoresis
CODIS	Combined DNA Index System
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
dsDNA	Double Stranded DNA
DO	Drop-Out (allele)
EDTA	Ethylenediaminetetraacetic Acid
ENFSI	The European Network of Forensic Science Institutes
EOF	electroosmotic flow
EtBr	Ethidium Bromide
FBI	Federal Bureau of Investigation
FTA	Flinders Technology Associates)
ILS	Internal Lane Standard
IWSTD	Irregular Warfare Technical Support Directorate
MDPD	Miami Dade Police Department
mtDNA	Mitochondrial DNA
NFSTC	National Forensic Science Technology Center

NGS	Next Generation Sequencing
OTS	Off the shelf
PBSO	Palm Beach Sheriff Office
PCIA	Phenol-Chloroform Isoamyl
PCR	Polymerase Chain Reaction
PEC-1	PCR Enhancer Cocktail
PHR	Peak Height Ratio
POP	Performance Optimized Polymer
RFLP	Restriction fragment length polymorphism
RFU	Relative Fluorescence Unit
RSID	Rapid stain identification-blood
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
SNP	Single Nucleotide Polymorphism
SPE	Solid Phase Extraction
SsDNA	Single Stranded DNA
STR	Short Tandem Repeats
SWGDAM	Scientific Working Group on DNA Analysis Methods
TAPS	3-{[1,3-Dihydroxy-2-(hydroxymethyl)-2-propanyl] amino}-1- propane-1-sulfonic Acid
Taq	Thermus Aquaticus Polymerase
TE	Tris-EDTA
TEM	Transmission electron microscopy

UV/VIS	Ultraviolet Radiation/ Visible
VNTR	Variable Number of Tandem Repeats
Y-STRs	Y-Chromosome STRs

CHAPTER 1 Overview

The goal of this thesis is to develop a microwave extraction protocol that can be coupled to a rapid and direct amplification method for the analysis of forensic samples.

Currently, there is a high demand for faster and more sensitive DNA results coming from the analysis of crime scene evidence (Butler 2015). Additionally, certain circumstances may require quick identification of a suspect, resulting in a need for developing fieldable techniques.

Common DNA extraction methods for body fluids are not suitable for field testing because they are not fast or cheap and may require toxic reagents that need to be handled in a chemical fume hood. Organic Extraction, the technique most used in laboratories is one example of this problem since several steps of the protocol require phenol-chloroform isoamyl alcohol. Other methods require large-scale robotic solid-phase extraction with magnetic beads and are not fieldable. Thus, an effective and fieldable method is needed to prepare and analyze forensic samples (Lee 2017).

Another critical issue in fieldable analysis is the condition of the evidence. The sample may be an aged, dried bloodstain contaminated with PCR inhibitors, or it might be a touch DNA sample with a very low amount of genomic content (Lee 2014). Such samples commonly require extensive laboratory workup, and the analyst must extract and process the evidence very carefully to avoid stochastic effects and inhibition, which can result in poor allele recovery (Giberson 1995).

For these reasons, we propose the use of microwave pretreatment followed by rapid direct PCR as a method to perform effective fieldable testing of a wide variety of forensic samples. Exposure to microwave radiation should increase the yield of DNA from

challenging samples that more typically require extensive laboratory workup. Ultimately, by increasing the yield of the genetic content of evidence in the field we can provide an optimal tool for the forensic analyst and assist with sample screening for criminal suspect identification.

1.2 Significance of the study and impact

This project represents a novel application of the microwave treatment for use in forensic DNA analysis. There are four main advantages of using this microwave extraction technique. First, it is fast. The method described in this project improves the recovery of alleles from crude samples requiring only 40 seconds of additional sample treatment. Standard extraction protocols can take hours to a full day to complete. Secondly this method is green and non-toxic requiring no harsh chemical or enzymatic treatments. In particular, toxic solvents such as phenol and chloroform are avoided. The process is also simple. Using a microwave does not require any specific training. Finally, microwave processing can be made portable and is not expensive compared to the costs of standard extraction kits.

Another unique aspect of this project is the development of a robust rapid direct PCR protocol. For the first time blood semen and saliva samples can be amplified without any chemical treatment, they are just exposed to the microwave treatment and directly amplified in less than 15 minutes using a custom master mix developed and optimized in this project.

This project impacts forensic science and nucleic acid analysis in several ways. It is known that biochemical extraction methods can lead to the partial loss of samples through

repetitive manipulation. Microwave processing is simple and minimizes sample manipulation. Finally, the microwave oven is portable and can be easily carried on site when there is a need for urgent analysis. The processes developed in this project can be easily extended to the medical field. For example, microwave treatment may be a useful step in a rapid preparation of viral saliva samples. A wide variety of medical and environmental assays could benefit from the coupling of microwave processing with direct PCR.

1.3 Dissertation structure

This dissertation is divided into 8 chapters. Chapters 1 to 5 provide information regarding the preliminary phase of the project in which saliva was chosen as a standard sample to initiate the optimization of the microwave pretreatment. Chapter 2 includes basic information on the development of forensic DNA analysis, the type of biological evidence included in the study, the DNA extraction techniques, and a literature review of Microwave extraction and rapid-direct PCR technique.

Chapter 3 describes the calibration tests that were performed using a commercial microwave oven. There were two goals for this process. First, it was important to select the proper locations in the microwave for efficient sample treatment. Several parameters were analyzed, including the effect of sample location on vial temperature and heating rate: Secondly, different power levels were examined to determine the most suitable irradiation energy to maximize the genetic yield. These parameters were then compared with those obtained from a specially designed laboratory-based, computer-driven microwave,

PELCO Biowave Pro + (Ted Pella Inc. Redding, CA)

Chapter 4 provides information about the content of saliva, the influence of dilution buffer, and conductivity effects. The quantification of saliva samples at certain dilutions using a different buffer and optimization of computer-driven microwave are discussed in Chapter 5. In Chapter 6 amplification and genotyping are discussed. A previously published protocol that uses a multiplex of 9 mini STR loci and a rapid thermal cycler was chosen as the basis to develop an optimized amplification and genotyping method for saliva samples. The resulting protocol was then extended to other body fluids, including semen, and blood. The last phase of the project, Chapter 7, describes collaborations with the National Forensic Sciences Technology Center (NFSTC), Miami Dade Police Department (MDPD), and Palm Beach Sheriff Office (PBSO) for additional validation of the procedure. The role of the external laboratories was to test the microwave extraction protocol on saliva samples collected on swabs, and directly amplify and genotype them using their RapidHIT instruments. The main goal of this collaboration was to corroborate the hypothesis of the project that microwave radiation could increase the yield and recovery of forensic DNA samples. Finally, a summary of the work with possible future lines of research is included in Chapter 8.

CHAPTER 2. Background and literature review

2.1 History of DNA analysis in forensic investigations

Due to the rapid evolution of the experimental techniques in molecular biology in the last twenty years, scientists can now discriminate individuals using the information contained in our DNA to a very high degree of selectivity (Butler 2009). Although it is well known all human DNA is more than 99% the same, several regions instead show different patterns between individuals. These sites are polymorphic, in that they present variations in the nucleotide sequence, or contain repetitions of some motif. Most of these polymorphic sites are located in non-coding regions of DNA, and for a long time, these regions were thought to be useless and were wrongfully called junk DNA because it was unclear what their function was, or if they had one at all (Palazzo 2014).

Human identification focuses on two main types of variation, sequence polymorphisms, and length polymorphisms. A particular type of sequence polymorphism is known as an SNP (Single Nucleotide Polymorphism), and it is characterized by a change in one nucleotide inside a genomic sequence. Over the past few decades, thousands of SNPs in both coding and non-coding regions have been identified and characterized for their relation to genetic disease and gene function. For example, certain SNPs are associated with increased breast cancer risk (Antoniou 2008). SNPs are also useful in forensic genetic casework and were among the first types of polymorphisms characterized by PCR (Pakstis 2010). However, the most useful DNA sequences for human identification are length polymorphisms, due to their abundance and high degree of variability between individuals. The earliest types of length polymorphisms used in forensic genetic studies were

minisatellites, also known as Variable Number of Tandem Repeats (VNTR), and Microsatellites, also known as Short Tandem Repeats (STR). The particular name, satellite, derives from the characteristic bands that this type of DNA creates following ultracentrifugation with Cesium chloride (Skinner 1977, Saul 1961).

Both kinds of polymorphisms present sets of nucleotides repeated in tandem. VNTRs are characterized by nucleotides repeated in sets ranging from 6 bp to 100 bp, and for this reason, they produce alleles that range in size from 600 bp to 30 kb. Conversely, STRs are shorter, containing tandem repeats in sets of 2–6 nucleotides, resulting in a much smaller set of alleles that range in size from 50 bp to about 500 bp.

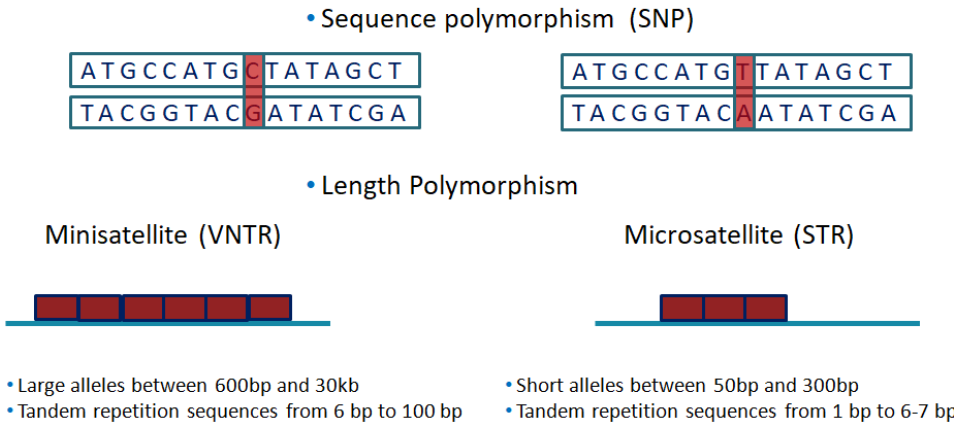

Fig.1 Schematic representation of the two types of polymorphisms in the human genome, sequence polymorphisms (SNPs) and length polymorphisms (VNTRs and STRs).

The first scientist that discovered these particular genetic features and used them as a tool for human identification was Sir Alec Jeffries. In 1984, he developed a technique for detecting VNTRs through a technique is known as Restriction Fragment Length Polymorphism (RFLP) detection (Jeffreys 1985); In this process, the genomic DNA is subjected to bacterial enzymes that cleave the sequence in specific regions, and the

resulting restriction fragments are separated using electrophoresis on an agarose gel. The fragments migrate along through the gel according to their length, so that the smaller fragments will migrate faster than the longer ones. In the following step, the fragments are transferred from the gel to a nitrocellulose membrane, just using its absorption characteristics. To help the transfer from the gel to the membrane a weight such as a book, is put on the top to facilitate the transfer and paper towels above the membrane absorb the buffer that transfer the DNA fragments from the gel onto the membrane by wicking. The membrane is treated to create ssDNA and radioactively labeled probes are added that hybridize to the single-stranded fragments containing the VNTR sequences. The resulting membrane is exposed to autoradiography film that permits the detection of VNTRs of interest.

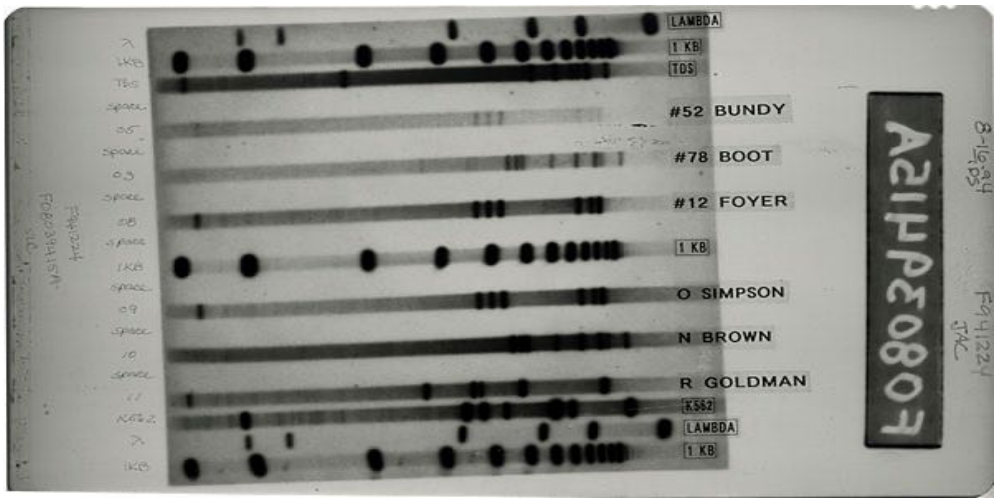

Fig 2 RFLP results in OJ Simpson casework. The autoradiography represents blood evidence collected and compared with the profile of the two victims and the suspect.
Image from https://mordundtotschlag.wordpress.com/2013/02/19/der-prozess-von-o-j-simpson/

Forensic scientists used this technique widely in fundamental research to improve the understanding of the behavior of those polymorphisms, as well as in forensic casework.

Figure 2 shows the RFLP analysis results on the DNA evidence collected in a famous crime scene, the murder of Nicole Simpson, ex-wife of former football champion OJ Simpson. In the autoradiography, the different pattern of the three individuals is evident, OJ Simpson, the suspect, and the two victims. The blood evidence that was collected and analyzed showed a match with the suspect that allowed the investigator to link OJ Simpson to the crime scene. Although the technique was the first in its genre and represented an incredible success for DNA typing analysis, it had certain limitations. This technique, unfortunately, required a relatively large quantity of good quality DNA, which is hard to obtain from forensic evidence, the procedure was lengthy, usually taking 4 days, and the handling of radioactive probes can be dangerous for the analyst (Butler 2009).

In 1983 the biochemist Kary Mullis invented the polymerase chain reaction (PCR), a method that dramatically changed the process of DNA analysis (Mullis 1986). The technique takes advantage of the inherent nature of double-stranded DNA to create a process to produce multiple copies of short specific sequences, using a single DNA template and a DNA polymerase. The amplification protocol is divided into three steps: denaturation, annealing, and extension (Figure 3). During the denaturation step that occurs at 95 °C, the two strands of DNA are separated. In the following annealing step, specific short sequences of oligonucleotide (20-30bp) known as primers bind to their complements on the two denatured strands at a temperature between 55 °C and 60 °C. The final step is the extension, made by a thermostable DNA polymerase enzyme that creates the two new strands of DNA starting the polymerization from the primers 3 prime end and incorporating nucleotides into the strands using dNTPs (deoxynucleotide triphosphate) This three-step cycle is repeated up to 30 times and generates hundred million copies of the DNA target.

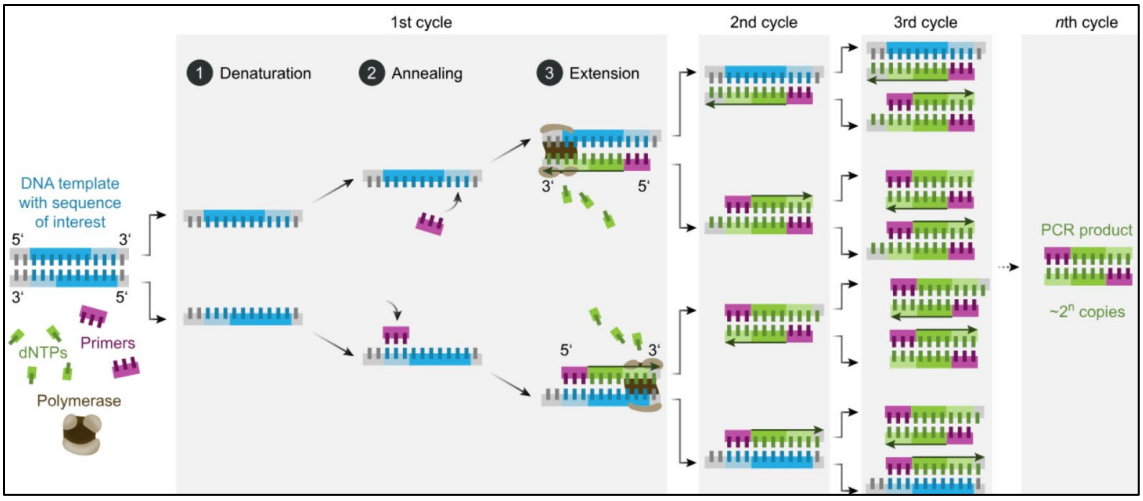

Fig.3 PCR amplification mechanism. The three steps are shown: denaturation, annealing and extension. In the first step of denaturation the sample is denaturated using high temperature usually around 95ºC for few seconds. Once the two strands are denaturated the primers can pair with the DNA at a certain annealing temperature. After the binding of the primers, the polymerase can start the extension step and polymerize the new DNA fragments that cycle after cycle will produce thousands of copies of the original DNA. Image from https://commons.wikimedia.org/wiki/File:Polymerase_chain_reaction-en.svg.accessed

The PCR greatly improved the sensitivity of detection and permitted the use of much smaller fragments of degraded DNA. As a result, all DNA analysis protocols for genetic and forensic casework that were in use before the introduction of PCR rapidly changed to include PCR as the initial step of the analysis. One of the earliest applications of PCR was the analysis of the VNTR D1S80, a minisatellite locus located on the first chromosome containing a 16 bp repeat unit (Duncan 1997, Budowle 1991). Detection was made through a silver staining technique that could be applied directly to the agarose gel (Duncan 1996). An example of this technique is shown in Figure 4.

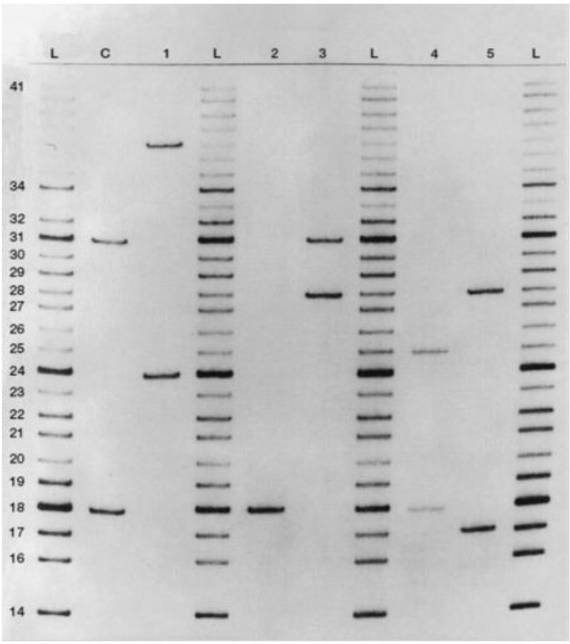

Fig 4. AMP-FLP results on D1S80 using silver staining technique (Schanfield 2000).
The lane C is the control, the L is the ladder, and the numbers represent the samples and their pattern.

A further and important improvement that allowed for shorter DNA analysis times was obtained by replacing minisatellites with microsatellite (STR) and running samples in horizontal acrylamide gels. Next, to improve information content and permit a greater degree of multiplexing, the PCR was performed using fluorescent dyes linked to the primers. Higher processing speeds and greater automation were obtained using a more modern separation and detection system, capillary electrophoresis (McCord 1993, Butler 1998).

STRs are the most widely used polymorphisms in forensic genetic analysis; they are short, easy to detect, and generate many alleles. In 1997, with the introduction of the first validated system by CODIS (Combined DNA integrated system), which used 13 STR loci,

scientists standardized a method for STR typing with high discrimination power (Budowle 1998). Moreover, through a series of genetic studies, an estimation of the allele frequencies was established for each set of STRs across population groups, creating a statistical basis for their use in forensic casework (Butler 2003, Park 2016). Currently, more than 24 autosomal STR loci are available for human identification, improving not only the power of discrimination but also the sensitivity and efficacy of the analysis. The smaller size of these STR amplicons makes it possible to also amplify old, degraded stains of semen or blood (Flores 2014). There are still issues to be solved with STR typing, as some sample types may be heavily degraded, at low copy, or combined in a mixture, creating results that can be difficult to interpret. The three examples of difficult samples are shown below.

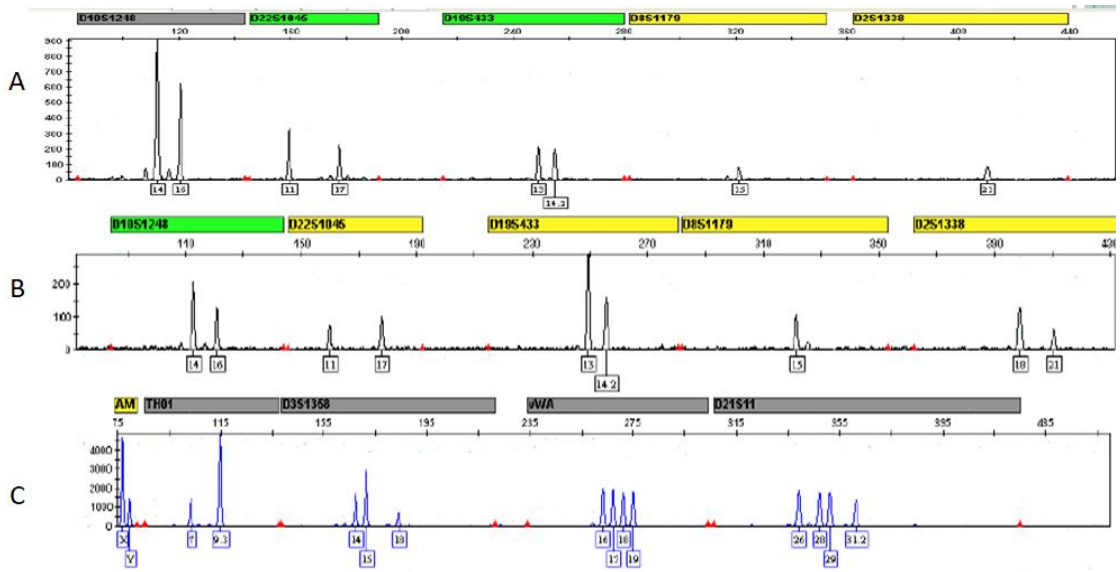

Fig 5 A, B, and C. Electropherograms showing three different DNA samples: A. degraded sample, B. low copy sample, and C. mixture sample. Saliva samples were prepared and treated from individual donors, collected on a buccal swab, treated, and amplified with the Investigator 24plex QS STR kit, Qiagen (QIAGEN inc, Valencia, CA).

Figure 5 A shows a degraded sample. It presents a distinct profile in which the short alleles have normal peak heights that slowly decrease as the alleles get longer in a well-known ski slope pattern known as curve decay. A result like the one cited above could lead to the inability to detect longer alleles, reducing the discrimination power of the assay. Unfortunately, degraded samples are very common and can result from human errors in the storage of the evidence, or through external effects, such as skeletal remains collected outdoors and exposed to the environment. A low copy sample involves evidence that contains less than 100 pg of genomic DNA (Martin 2016) and is subject to stochastic effects. These include problems such as peak imbalance, high stutter, allele drop-in, and allele drop out, which make interpretation very difficult (Budowle 2009, Gill 2000).

Mixtures represent another type of problematic DNA sample. Examples might include vaginal swabs collected after a rape, in which the sample contains a mixture of both male and female DNA, in which the female DNA is much more abundant than the male. Complex mixtures may contain 3 or more individuals in a profile. For this reason, the data interpretation for these particular samples is very difficult, although recommendations on how to interpret those results have been established (Gill 2006). Forensic scientists have tried overcoming these issues by implementing new tests. For example, developments such as the application of Mitochondrial DNA (mtDNA) and the use of polymorphisms on the Y chromosome (Figure 6) can greatly assist with low-level samples and the analysis of rape kits (von Wurmb-Schwark 2009, Kayser 1997). Every eukaryotic cell contains hundreds of mitochondria, and thus, hundreds of mitochondrial DNA copies. This type of DNA can therefore be exploited as an alternative method for the analysis of highly

degraded samples, in which the autosomal DNA is at levels too low to amplify. Mitochondrial DNA contains two particular regions known as hypervariable regions 1 and 2; together, these form the D-loop, which is highly polymorphic as it is non-coding. Therefore, this area of the genome is a useful target for human identification. Because Mitochondrial DNA is inherited via the maternal germline, with no paternal contribution, the use of mtDNA is also very useful for ancestral and familiar studies. Synergistically, the Y Chromosome can be useful as it is inherited through the paternal germline and contains extensive information useful in forensic studies, such as short repetitive sequences and SNPs, which can be used to support autonomic analysis, especially in hard to interpret samples, such as mixtures (Buckleton 2011).

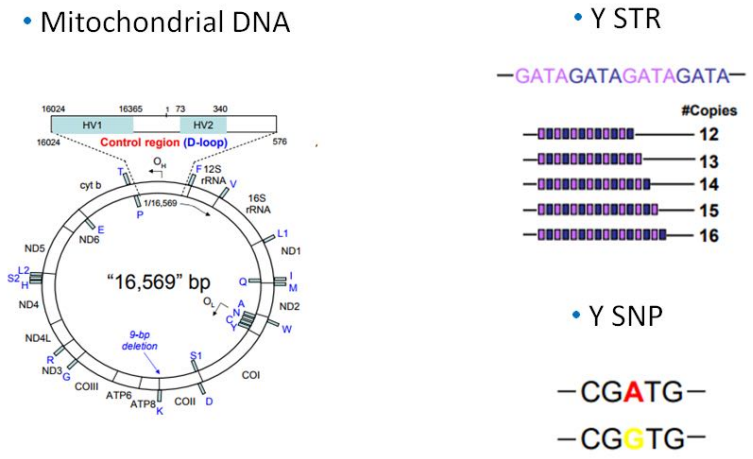

Fig 6. Mitochondrial DNA structure (left) and examples of polymorphisms on the Y chromosome (right) (Butler 2005).

Recently, new applications have been introduced in forensic analysis to increase the discrimination level between individuals and improve forensic DNA analysis (Bornman 2012). One of these techniques is known as NGS (Next Generation Sequencing). This sequencing method has recently been applied in STR and SNP genotyping (Børsting 2015). Nowadays, several platforms are available with different sequencing and detection

methods. One of the most currently used is the Illumina system (Verogen Inc., San Diego, CA).

This company has produced several forensic panels allowing analysts to sequence STRs, SNPs, and other genetic elements such as mitochondrial DNA, X, and Y chromosomal DNA (Xavier 2017). The sequencing reaction occurs through a synthetic process in which a fluorescent detection step following the addition of each base (sequencing by synthesis). DNA samples coming from different individuals can be analyzed and identified simultaneously, thanks to an artificial barcode applied by the analyst during the sample preparation phase. Another interesting sequencing technique that has been recently applied in forensics uses nanopore membranes (Feng 2015). Nanopore membranes resemble membrane proteins that are natural channel-forming pores and are present in biological cells. Under appropriate experimental conditions these pores to allow for the transmission and detection of nucleic acids upon application of a voltage (Kasianowicz 1996). Following this general idea, Oxford Nanopore technology created a device, the MinION, which is small as a phone and can sequence longer strands of genomic DNA. The device can be used in large scale detection of SNPs and STRs in extracted DNA (Cornelis 2017). Despite sequencing being a valid and efficient technique, it's a high throughput but not rapid method, and the protocol includes many steps that increase manual errors and slow down the analysis.

Currently, there are a number of newly developed automated DNA analyzers which focus on detection of oral fluids on cotton swabs. These systems are mainly designed to be used in police stations and or the field and require only minimal sample pretreatment. Unfortunately, these methods target saliva and lack the sensitivity and flexibility required

for the analysis of more complex samples, such as blood and semen. Moreover, while these instruments are known as rapid DNA analyzers the actual procedure is not that rapid, requiring 90 minutes to produce a profile. Therefore, there remains a need to develop a fast and reliable method to quickly process samples coming from crime scenes.

2.2 Biological Evidence

At the crime scene, different types of biological evidence are collected. Unfortunately, for many types of DNA evidence, extraction and analysis are complex processes. For this reason, it is important to develop and apply clear guidelines for the collection and preservation of evidence prior to laboratory analysis. This process, involving evidence collection and preservation of chain of custody has been established to maintain the integrity of what is collected at the crime scene. Specific, preestablished protocols are used to avoid damage and minimize the risk of improper handling or contamination (Tomlinson 2006). In this section, we will introduce the three different biological evidence types used in this research project.

2.2.1 Saliva

Liquid saliva represents the most commonly used body fluid for DNA typing analysis, paternity testing, and sample reference collection. While it is possible to find saliva on many items and materials, it is often hard to visualize. For this reason, first responders often use a forensic light source to analyze the area in which the crime occurred for the detection of trace evidence. Saliva, semen, and vaginal stains naturally fluoresce, each at a

specific wavelength. For example, saliva can be detected at wavelengths around 470 nm (Camilleri 2006).

Once detected, it can be collected using a cotton swab with a double swabbing technique (Pang 2007) and tested using a presumptive test. The Phadebas® test (Phadebas Inc Cambridge, MA) can also be used to detect saliva (Hedman 2011). Amylase is an enzyme that is present at very high concentrations in saliva, and it catalyzes the hydrolysis of starch (Latin amylum) into sugars (Whitehead 1975). The test is performed using paper on which starch bound to a dye has been previously deposited. If amylase is added, the digestion of starch occurs and the blue dye is released, creating a color change.

2.2.2 Blood

Blood evidence can be liquid, dried, or latent. Fresh blood can be collected directly onto a swab and let dry before sealing it in a paper bag. Dried bloodstains instead need to be first scraped from the support using a knife, and then collected in a paper bag or alternatively collected using a moistened swab, dried and then collected in paper.

Trace blood evidence can be difficult to detect if attempts have been made to clean the area. Here, the investigator can utilize luminol to detect the stain (Advenier 2018).

Luminol (5-amino-2, 3-dihydro-1, 4 phthalazine dione) is widely used as a presumptive test in forensic analysis. The target is hemoglobin, a protein responsible for transporting oxygen in the blood. In the presence of an oxidant, the heme group in the protein can catalyze a chemiluminescence reaction with luminol. (Blum 2006) Unfortunately, this reaction can give false-positive results, as luminol can react with copper and iron

compounds as well. For this reason, it is common to first use an alternative light source at 900nm, to detect the stain (Advenier 2018).

Another common presumptive blood test is the Kastle-Meyer test. It is based on an oxidation reaction between phenolphthalein and oxygen peroxidase in the presence of blood. The reaction turns pink upon reaction with hemoglobin. (Kastle 1901, Meyer 1903, Virkler 2009). Once detected, blood stains can be confirmed by a number of more specific tests. For example, the Rapid Stain Identification of Human Blood (RSID™Blood) is a monochromatic test that is contained in a cassette strip similar to Covid19 antigen tests. The target of the confirmatory test is glycophorin A, a red blood cell membrane antigen that is common in human blood. Two monoclonal antibodies react when the glycophorin is detected and the positive reaction is visualized by a red-colored band that appears on the strip (Turrina 2008) (Figure 7).

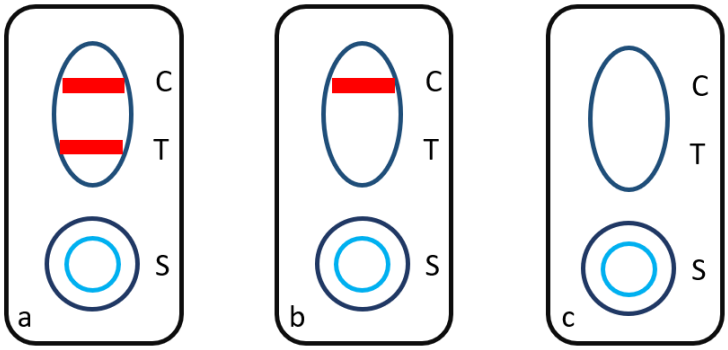

Fig. 7. Schematic of a RSID blood test. The sample is introduced in the sample (S) window, C is the control lane, T is the test lane.Three are the possible outcomes; 7a positive result, 7b negative result or 7c inconclusive result. Image created by the author

2.2.3 Semen

Semen evidence is also detected with alternative lights followed by presumptive and confirmatory tests. Both UV light and/or a Polilight® (450 nm) can be used to identify semen stains present on a range of different materials (Vandenberg 2006).

The classic presumptive test for semen is the acid phosphatase test (AP). Acid phosphatase is an enzyme in semen that can react with a substrate such as a-naphthyl phosphate in the presence of Brentamine fast Blue B, changing the color of the solution to purple (Raju 1964). Since Brentamine Fast blue is a carcinogenic reagent, forensic scientists must be careful while performing the test. Recently an alternate method to detect both semen stain and saliva has been developed. In many sexual assault cases, the perpetrator can leave either semen or saliva evidence that needs to be analyzed avoiding any loss of genetic content. This new method couples the AP test to the Phadebas® filter paper. The results obtained using this strategy demonstrated that semen and saliva can be detected without affecting the condition of the stain, such that successive typing studies can be conducted, showing that this method can be a powerful tool for sexual assault cases (Herman 2018).

Several confirmatory tests have been developed to detect semen, the simplest of which is microscopy. Other antibody-based tests have also been developed, such as the RISD for semen. This lateral flow strip test targets semenogelin protein, whose presence is detected by its attachment to two monoclonal antibodies (Old 2012.).

2.3 DNA Extraction

Nucleic acid extraction is a fundamental step in all biological analyses and is particularly important in forensic DNA analysis. Several types of DNA extraction techniques are available depending on the sample type and form of DNA recovered (Lee 2017). In forensic DNA analysis the extraction step is critical because, recovery can vary with sample type, condition and the presence of PCR inhibitors (Demeke 2010).

The most common manual method used is an organic extraction procedure which is based on **Phenol-Chlorophorm** treatment (Figure 8). The treatment separates cell debris into organic and aqueous phases, allowing for the isolation of DNA (aqueous phase) from proteins and lipids (organic phase). Although it represents a valid extraction technique, the reagents that are employed are toxic, the procedure typically takes two days, and it is not suitable for all types of forensic evidence (Comey 1994).

Another method utilizes proteinase K to hydrolyze the cell membrane, followed by the addition of salt to isolate and purify the nucleic acid. This extraction method can provide high yields of DNA, but if salts are not carefully removed, they will interfere with the successive downstream amplification steps and methods (Grimberg 1989, Aljanabi 1997). Another valid extraction method involves boiling the sample in the presence of **Chelex ® 100,** an ion exchange resin that is added directly to the samples, trapping magnesium ions and inactivating the nucleases, while keeping the DNA protected. While this method is suitable for extracting blood and semen, if the amount of blood is too high, inhibition may be produced in the PCR (Walsh 2013).

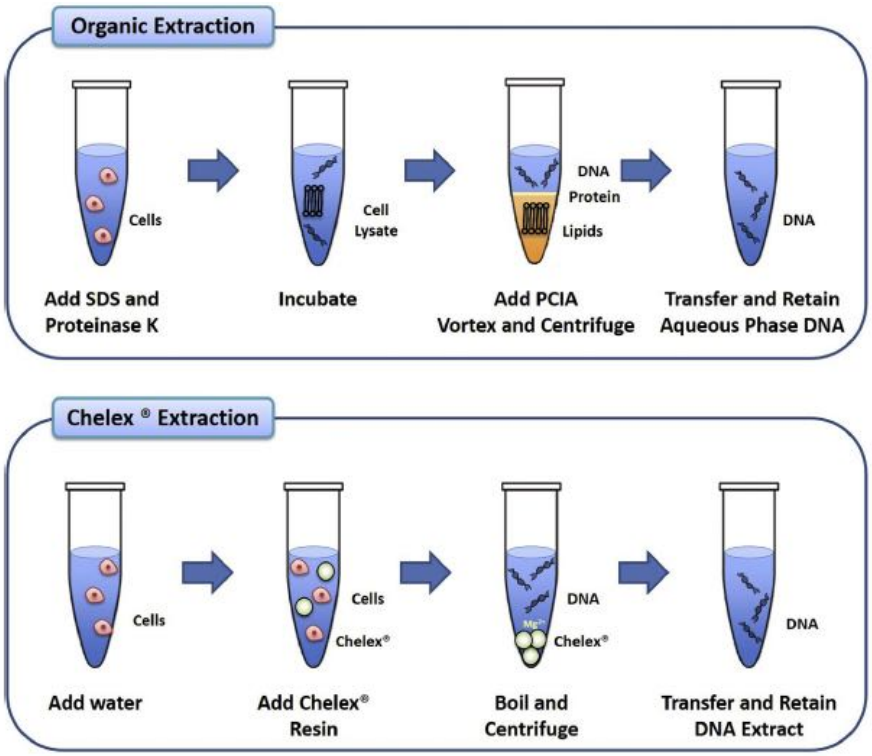

Fig. 8. Organic and Chelex extraction mechanism
In the organic extraction (top) the sample is treated with a detergent, sodium dodecyl sulfate (SDS), and Proteinase K to lyse the cells, after an incubation step, Phenol-Chlorophorm is added. The following centrifugation step allows the separation of the sample in phases. The nucleic acid if interest will be found in the aqueous layer. Once removed it can be transferred and retained into a clean tube. In Chelex extraction (bottom) the sample is boiled with water and Chelex resin. The resin binds to the Mg^{2+}, leaving the DNA in the aqueous solution ready to be transferred and analyzed (McKiernan 2017).

Differential extraction. It is very common in a crime scene to collect evidence involving a sexual assault. This evidence is usually represented by a mixture of several body fluids, such as semen, saliva, or vaginal secretions, that together are hard to analyze. The result is a mixture of 2 or more people, and in the case of a sexual assault, there is a need to isolate the male and female components of the mixture. The original differential extraction method was created by Peter Gill more than three decades (Gill 1985). The procedure consists of two different lysis steps. In the first, the non-sperm cells are treated with a detergent (sodium dodecyl sulfate, SDS) and Proteinase K to be lysed. The treatment does not affect sperm cells, because of the strong disulfide bonds between proteins contained in the sperm heads, where the nucleus is contained. The sample is then centrifuged so that the non-sperm fraction can be collected as a supernatant layer, while the sperm cell is collected as a pellet. The DNA from the non-sperm cells is then extracted using the common organic extraction method Phenol-Chloroform.

In the next step, the sperm fraction is lysed by detergent and treated with DTT to reduce the disulfide bonds and release the nucleic acids. After an incubation period of 2 hours, the male DNA is extracted using phenol/chloroform (Gill 1985). Although this method is widely used, it has some limitations. It is a very time-consuming procedure and during the washes to remove the victim's DNA from the pellet, there can be a sperm loss (Hudlow 2012).

More recently an innovative method for the extraction and preservation of forensic evidence was introduced, **FTA paper**. FTA paper, as the name suggests, is filter paper that contains reagents on the surface, which can trap inhibitors and contaminants and inactivates microbial growth. The evidence technician collects the evidence on a swab, and after

hydrating the swab with water, they can rub the paper to deposit the sample on the filter. This method is particularly useful for collecting evidence on site (Nuchprayoon 2007).

Parallel to the technological advances in DNA analysis, extraction techniques were improved as well, to respond to a pressing need to make this step easier and faster.

Solid phase extraction (SPE) techniques enable the DNA to bind to a solid surface, silica resin or magnetic beads, in the presence of high salts concentration. Briefly, a lysis buffer is added to the sample, once the cell is lysed, silica paramagnetic beads can be used to attract the DNA to their surface, and a magnet can be used to keep the DNA bound while the other components can be washed away. The DNA isolated can be transferred and eluted and used for further analysis (Figure 9) (McKiernan 2017).

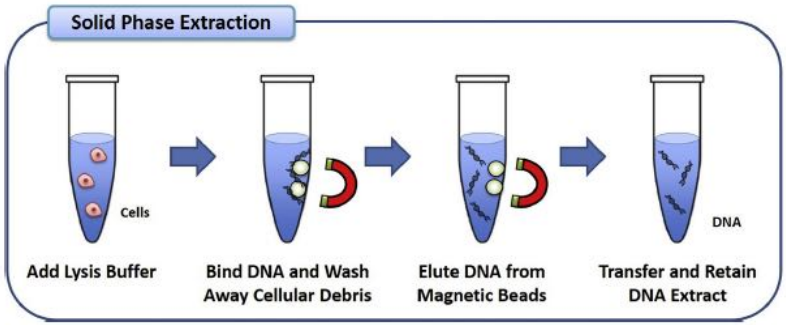

Fig. 9. Schematic of Solid Phase Extraction (SPE).
The sample is treated with a lysis buffer and paramagnetic beads are introduced. In the presence of high salts concentration, the DNA will bind to the beads. A magnetic stand is used to keep the DNA bound. Several washing steps are included to clean the sample from cellular debris. Once done with the cleaning step, the DNA can be eluted and retained for further analysis (McKiernan 2017).

Several commercial kits based on SPE are available. DNA IQ™ kit (Promega Corporation, Madison, WI) uses paramagnetic resin beads. The sample is treated with lysis buffer and after an incubation period the resin with the beads are introduced in the sample. The tube is positioned into a magnetic rack stand that allow the immobilization of the DNA. The

following wash steps can occur in the same tube as long as it remains in the magnetic rack. After the washes the DNA can be collected from the tube (Mandrekar 2001).

QIAamp® Kit (QIAGEN inc, Valencia, CA) employs spin columns with silica gel membrane to bind the DNA. The mechanism is similar to all the other SPE-based kits. The first phase of lysis is followed by the retention of the nucleic acid onto the silica membrane, then two washing steps with different buffers allow the purification and elution of the DNA (Greenspoon 1998).

Another kit to mention is The PrepFiler™ from Thermo Fisher Scientific (ThermoFisher, Foster City CA) The isolation of DNA occurs on magnetic resin similarly to other commercial products, the difference is in the coating of the beads that is not silica but a derivative of dextran and the presence of an alcohol, isopropanol in the binding solution (McKiernan 2017, Brevnov 2009).

Most of these kits can be used in **automated extractor systems**, such as the Robot EZ1 from Qiagen (Qiagen, Inc., Valencia, CA) or Maxwell 16 (Promega Corporation, Madison, WI). The main advantage of using an automatic extraction is the ability to minimize contamination between the various steps of an extraction protocol. The robot uses prefilled cartridges that are sealed and introduced in the instrument without being opened. Moreover, the SPE kit provides a high yield and purity of the extracted DNA, making the automated process very valuable (Krnajski 2021).

In modern forensic DNA analysis, one of the main goals is to establish a reliable protocol that encompasses the collection of evidence, extraction, amplification, and typing, in which the inhibition is minimized, sensitivity is improved, and the whole process is faster than

previous methods. To this end, the introduction of a microwave step before amplification could represent a critical positive change in this direction.

2.4 Microwave Extraction

Microwaves belong to the electromagnetic radiation spectrum and range between 1 mm and 1 meter (Figure 10). The radiation can be continuous or pulsed, depending on the intended use. Continuous microwave radiation is widely used in communications, while pulsed microwaves are found in medical and domestic applications ("NASA 2010).

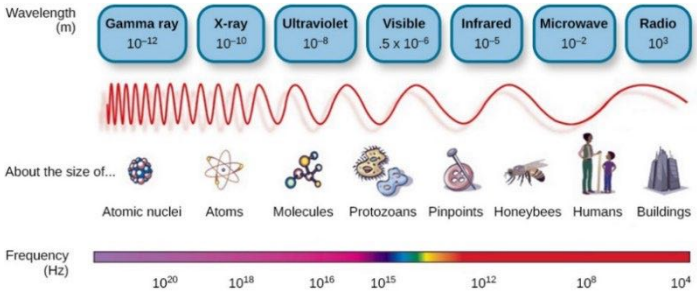

Fig.10 Electromagnetic spectrum. Reproduced from NASA http://science.nasa.gov/ems/06_microwaves

Inside the microwave oven, there is a device called a magnetron, which is responsible for the production of microwaves. Once they are generated, the radiations are reflected within the metal interior of the oven, where they are used to heat food (Figure 11). The heating process results from the absorbance and excitation of water molecules in the food. Microwave radiation causes water molecules to rotate, due to their asymmetrical distribution of charge. The rotation of the water molecules and collisions with other water

molecules results in a transfer of kinetic energy that increases the temperature, producing heat that penetrates the food (Malheiro 2011).

Inside the microwave oven interactions between various waveforms results in a phenomenon called interference. There are two types of interference, constructive and destructive. The first occurs when the sum of the resulting electric field from the interactions of the microwaves at some point is greater than the singular electric field generated. Therefore, the molecules have more kinetic energy, generating more heat. In contrast, destructive interferences generate a null electric field, reducing the intensity of the waveform and therefore causing the food to be poorly heated. To avoid this uneven heating, rotating plates were introduced in all microwave ovens (Halliday 2010).

In the early ’70s, scientists were already studying microwave features, trying to understand how to apply them for research on biological systems (Har-Kedar 1976).

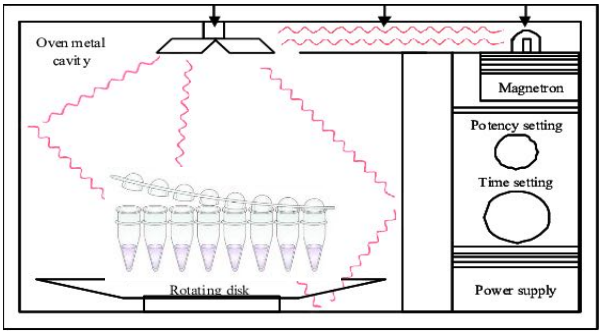

Fig.11 Microwaves radiation path in a commercial microwave oven.
In the figure is shown the possible radiation path and the tubes with biological samples that can be introduced in the microwave and exposed to the radiation. Edited from Malheiro (2011)

2.5 Literature review

Microwaves have often been used in the study of biological systems, due to their ability to improve the dissolution of lipids and other organic molecules present in the cell. (Mukherjee 2019) (Figure 12). Microwave radiation disrupts the membrane of the cell so that the internal material can be exposed without requiring the use of any detergent or chemical substance (Mukherjee 2019, Tsubaki 2017). As an extraction technique, it has been widely used and optimized to recover specific components from food such as pectins, polyphenols, and flavonoids (Ekezie 2017).

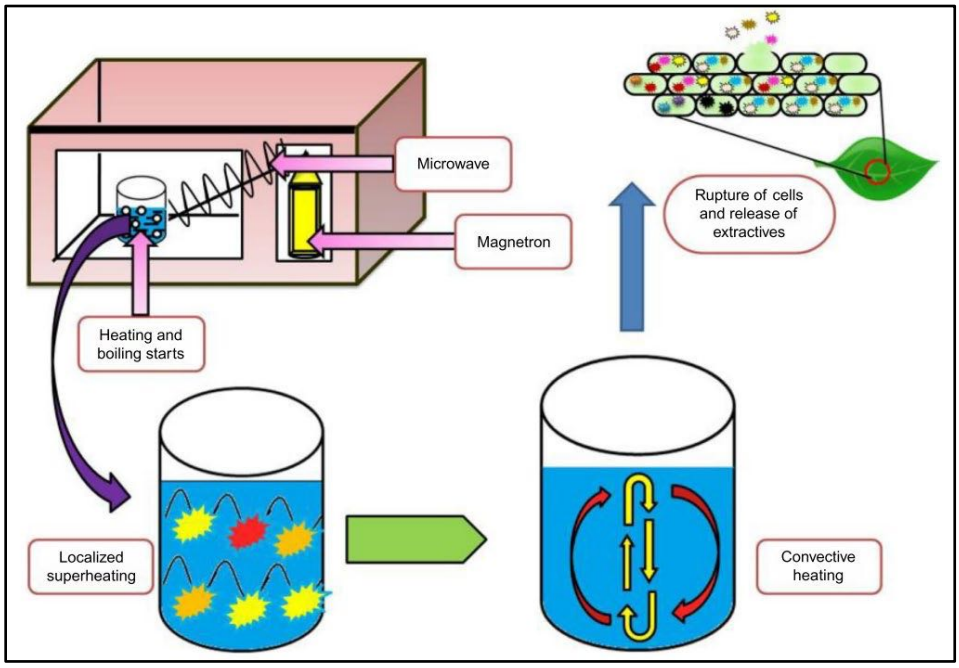

Fig.12 Microwave exposure effect on plant cells. The exposure to 15-30 min of microwaves, in the example provided, allows the large recovery of analytes when compared to common methods of extraction (Mukherjee 2019).

Numerous studies have been published on the use of microwaves as a DNA extraction technique for plants (Goodwin 1993), bones (Imaizumi 2013), environmental samples (Orsini 2001), and in the medical diagnostic field (Melendez 2016).

In pathology, it is very hard to efficiently extract DNA from aged formalin-fixed samples because they are low in DNA content. A slow tedious procedure is often required. Wu et al. reported that exposure of a formalin-fixed tissue sample to microwave irradiation led to a significant increase in the yield of DNA compared to non-microwaved samples (Wu 2012).

One of the first studies that employed microwaves for DNA extraction purposes, focused on the analysis of different types of gram-positive and acid-fast bacteria. In the study, several standard approaches were attempted to isolate DNA from the bacteria, however, it was impossible to extract the DNA of bacterial strains from *Mycobacterium tuberculosis* and *Corynebacterium xerosis*. However, the use of a microwave oven, as shown in the table, allowed DNA extraction of all bacterial strains. Moreover, an increase in the yield of DNA after microwave treatment was observed for all organisms under study (Bollet 1991) (Figure 13).

Bacterial strains	amount of DNA (μg/200 mg of wW)	
	3% SDS	3% SDS + MWO
Mycobacterium tuberculosis	0	50
Actinomyces israelii	10	200
Nocardia asteroides	10	100
Streptococcus pyogenes	50	200
Rhodococcus equi	5	50
Bacillus gordonae	5	50
Corynebacterium xerosis	0	20
Clostridium perfringens	20	150

Fig.13. Microwave extraction method applied to several bacterial strains. The results demonstrated that DNA yields increased after microwave treatment compared to the standard extraction technique (Bollet 1991).

Another study directly compared commonly used extraction methods, including chemical and enzymatic methods, to microwave-assisted extraction on Gram-positive and Gram-negative bacteria.

As a chemical treatment, the samples were boiled and then extracted using phenol-chloroform. The enzymatic protocol included proteinase K and SDS, followed by phenol-chloroform extraction. The microwave treatment instead included a wash with SDS followed by two periods of exposure to the microwave. The resulting extract was next extracted using phenol-chloroform.

After the treatment, the samples' quality was assessed with agarose gel, UV/Vis spectroscopy, and PCR. The results in Figure 14 showed that the use of microwave irradiation (1 hour DNA extraction procedure) significantly increased the DNA recovery and purity over the other methods (Omar 2014).

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Table 1. Concentration and purity of bacterial MRSA and ESBL DNA isolated by three DNA extraction methods.

Microorganism	DNA Concentration µg/ml			A260/A280		
	Boiling	Enzymatic	microwave	Boiling	enzymatic	Microwave
MRSA (Gram-positive)	71.0	61.8	80.3	1.21	1.75	1.81
	71.5	62.2	79.9	1.23	1.76	1.82
	71.1	62.3	80.1	1.22	1.80	1.83
Mean ± S.D.	71.2 ± 0.26	62.1 ± 0.26	80.1 + 0.2	1.22 ± 0.01	1.77 ± 0.03	1.82 ± 0.01
ESBL (Gram-negative)	65.4	55.6	91.4	1.16	1.56	1.68
	65.7	55.2	91.0	1.15	1.54	1.72
	65.4	55.7	91.2	1.20	1.55	1.70
Mean ± S.D	65.5 ± 0.17	55.5 + 0.26	91.2±0.2	1.17 ± 0.03	1.55 ± 0.01	1.70 ± 0.02

Fig.14. Comparison of DNA yields and purity using Microwave irradiation vs Boiling and Enzymatic Extraction. For both bacterial strains, microwave extraction produced an increase in both yield and purity (Omar 2014).

A very interesting study focused instead on the effect and ability of microwaves to function as a sterilization process for a common spore present in raw meat, *B. cereus*. The bacterial strain was subjected to several pretreatments before being exposed to microwave irradiation for 5 minutes with a power of 1800 W at 85 °C, as measured by an infrared probe. After exposure, the bacterial strain was analyzed to determine if any changes occurred in its morphology, its nuclear chromatin, or protein expression.

The results obtained by Scanning Electron Microscopy (SEM) clearly showed that microwave treatment altered the morphology of *B. cereus* cells, breaking their membrane. furthermore, 18 of 23 proteins identified were down-regulated and 5 were up-regulated. Therefore, microwave treatment also affected the metabolic pattern of the bacteria.

Finally, Transmission Electron Microscopy (TEM) imaging confirmed the ability of microwaves to alter the morphology of these spores and permeate their cell membrane, releasing nucleic acid and inactivating the spores (Figure 15) (Cao 2018).

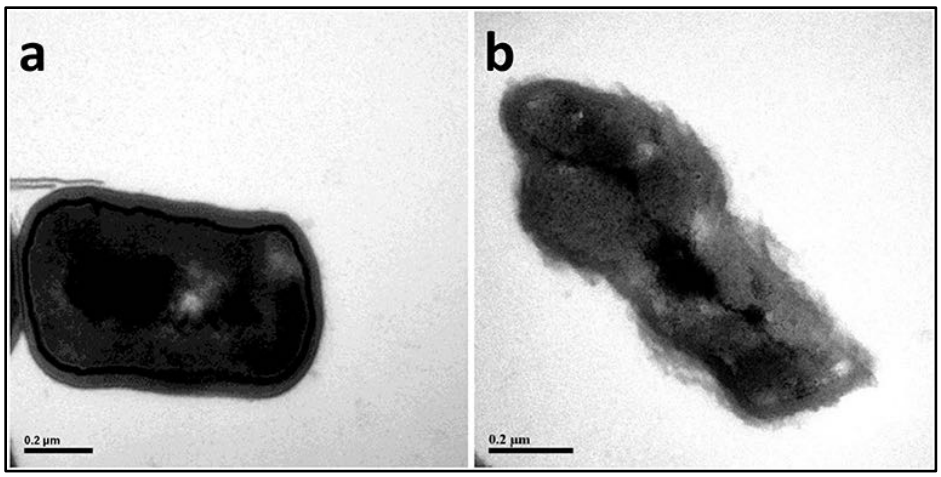

Fig.15 The transmission electron microscope (TEM) photography of Bacillus cereus in the control group (× 2,500) (a) and in the treated group (× 2,500) (b), respectively. The effect is clear, the microwave treatment inactivated the spore (Cao 2018).

While many studies have been conducted using microwaves as an extraction tool for broad biological or medical applications, less research has been conducted on forensic applications. Some of these studies performed in 1995, compared extraction methods involving computer-driven and commercial microwave ovens with extraction by Phenol-chloroform DNA extraction (Giberson 1995). Analysis by gel electrophoresis of a variety of DNA extracts (Figure16) showed that the efficiency of the microwave-assisted extraction was as good as standard organic extraction methods, and reduced the time for extraction for blood, saliva, semen, skin, fingernails, and hair in forensic analyses from 2 hours to 2 minutes (Giberson 1995) and microwave extracted DNA samples have been shown to be compatible with downstream PCR (Thomas 2018).

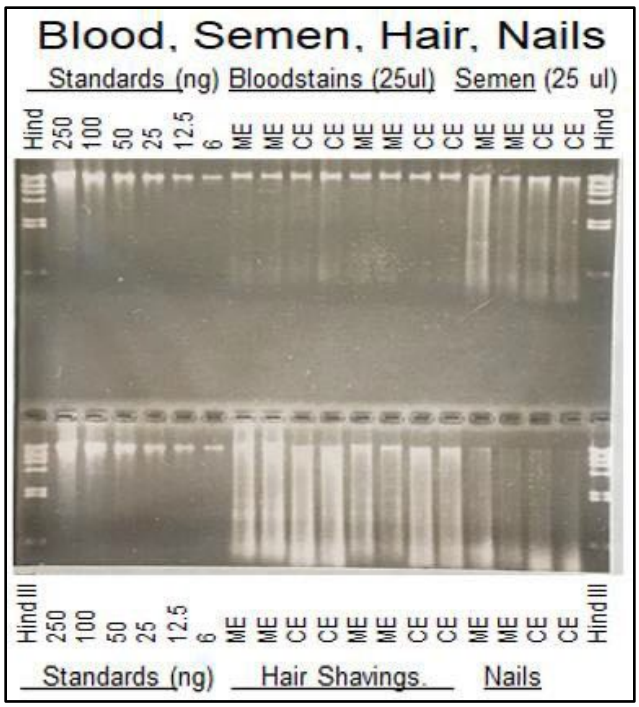

Fig.16 Electrophoretic gel showing a comparison between common extraction (CE) and Microwave extraction (ME) for a variety of substrates. The results for ME extraction are comparable in quality and quantity to those observed for CE extraction (Giberson 1995, Thomas 2018).

2.6 DNA Amplification

In this chapter two different approaches to DNA amplification will be discussed.

The first section includes the development of rapid PCR techniques, the ability to speed up the amplification reaction without affecting the efficiency of the DNA analysis, and the application of this technique in forensic science.

The second approach involves Direct DNA analysis. This methodology allows the scientist to work with crude samples and to skip the extraction step. Despite the difficulties of amplifying a non-treated sample, researchers have been able to develop and optimize new protocols and adapt them to the technique.

Lastly a brief section will be dedicated to the application of both direct and rapid DNA analysis which provides a powerful tool for processing crime scene evidence.

2.6.1 Rapid PCR

Not so long after the discovery of the PCR by Kary Mullis (Mullis 1986), the scientific community began investing their efforts in the improvement of PCR performance, particularly focusing on the kinetics and speed of the process. Among the earliest researchers studying this process was Carl Wittwer who focused on improving the speed of the PCR (Wittwer 1991). In particular he worked on optimizing instrument methods by improving heat transfer, introducing capillary tubes for rapid heat transfer, and developing a rapid thermocycling device. The results obtained from his tests showed that faster reactions did not affect the quality of the amplification products.

Figure 17 shows the results of different amplification protocols, all of which were set up at 30 cycles, as well as the agarose gel that was run on to test the quality of amplification products. A 536 bp β-globin protein fragments was amplified using two different PCR protocols, a standard method that employed a common thermal cycler with microfuge tubes, and a faster protocol using a rapid instrument and capillary tubes. The standard protocols in Figure 17 A and B, required 4 and 2 hours, respectively, giving non-specific products on the gel, while the rapid protocols in C and D required 40 and 15 minutes, and amplified the fragment with no specific bands on the gel. Wittwer reported that product specificity was achieved using a denaturing step at 93 °C and then annealing at 55 °C for less than 1 second.

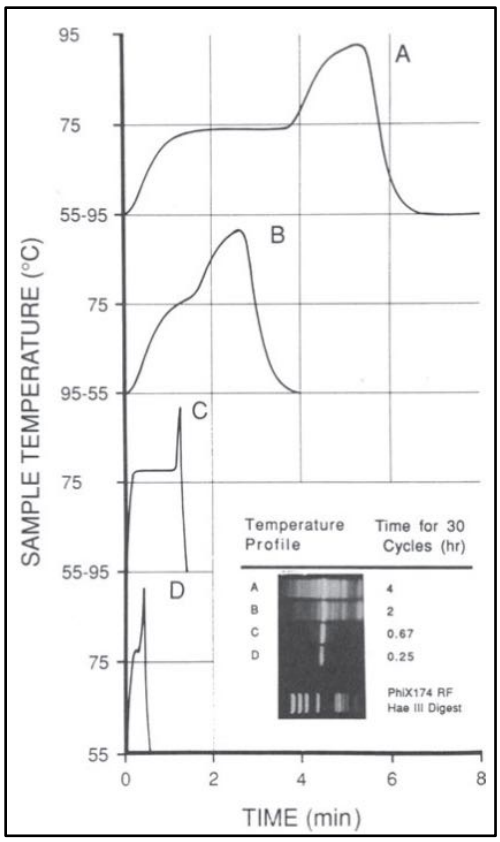

Fig. 17 Comparisons between standard thermal cycler (A, B) and rapid instrument (C, D). A β-globin protein of 536bp was amplified according to different amplification protocols. The results in A and B using standard protocol (4 and 2 hours respectively) showed the presence of non-specific products on the gel, while faster protocols, C, and D performed in 40 and 15minutes showed clear bands on the gel. (Wittwer 1994)

Figure 18 A, B, C, and D describe results on the optimization of parameters for rapid amplification by Wittwer (Wittwer 1994). In Figure. 18A, the annealing time parameters are analyzed. The results show that if the annealing occurs for less than 1 second, the specificity is high. It is clear instead from the gel that a longer annealing time creates non-specific products.

Figure 18B demonstrates the effect of elongation time. In general, short elongation times do not appear to reduce yield, at least for this fragment size.

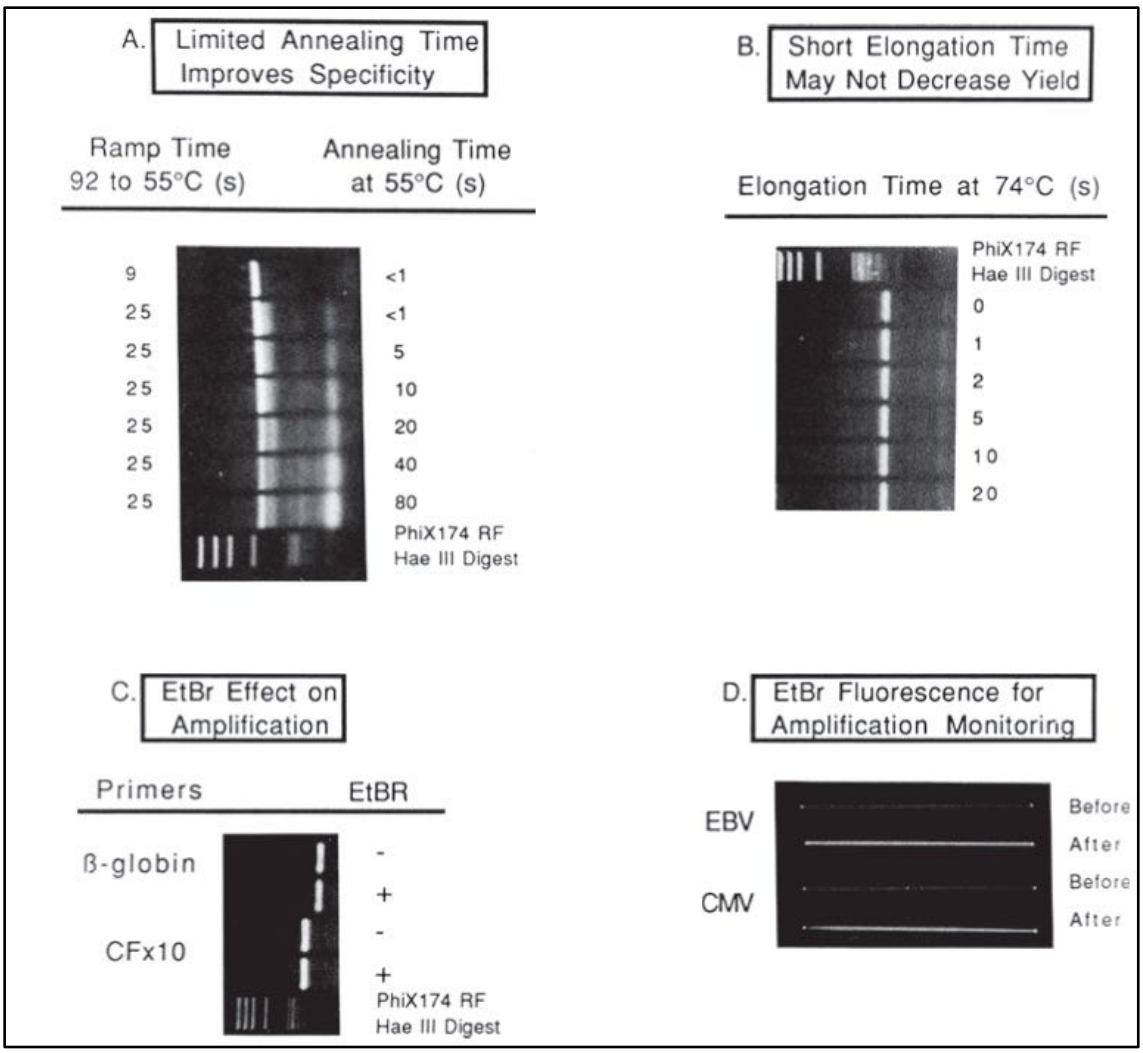

Fig 18 A, B, C, and D. Optimization Rapid amplification protocol.
The effect of several parameters' optimization is shown. In 18A the limit annealing time, less than 1 sec provided higher specificity. The effect of elongation time in 18B demonstrated that short elongation time can be used without affecting the results. In 18C and 18D the effect of EtBr on the amplification process is shown. The results suggest that the use of EtBr does not affect the amplification (18C) and can be used to monitor of PCR performance (18D) (Wittwer 1994)

In Figure 18 C the agarose gel shows the results of the amplification of β-globin fragment under two conditions, regular amplification and amplification with ethidium bromide (EtBr) incorporated directly in the PCR reaction. This experiment was useful to demonstrate that ethidium bromide did not affect the amplification reaction, nor the yield

of DNA obtained. Figure18 D shows the effect of adding EtBr before the amplification and after the amplification. This technique, according to Wittwer, could be a very useful tool to monitor the performance of the PCR and control how many cycles are enough for a target fragment to be fully amplified.

Following Wittwer's experiments, several other research projects have been focused on rapid DNA analysis, and the interest in this new technique for forensic DNA applications has substantially increased.

Some studies focused on developing a faster protocol for ABO blood typing because it's very useful for forensic identification purposes, however it is a process that has a lot of steps and it's not fast (Lee 2011). Other researchers developed multiplex PCR on a microfluidic device in order to speed up the process of amplification and typing (Estes et al. 2012). Components of the standard STR kits needed to be improved, and more efficient DNA polymerases and cycling conditions needed to be tested and optimized. To this end, new kits were developed capable of completing a PCR amplification in less than one hour. Several studies developed new protocols; one is represented by the work of Dr. Laurin. The goal was to achieve a rapid amplification of forensic samples using a commercial kit the Amp/STR® Profiler Plus® STRtyping under 30 minutes. This group was able to amplify forensic samples in less than 26 minutes by modifying the cycling parameters, the thermal cycler instrument, the polymerase, and its buffer (Laurin 2012).

Additional studies demonstrated the efficiency of the rapid PCR amplification by selecting different thermal cycler instruments with novel parameters including, cycling time, DNA polymerases and buffers (Romsos 2015).

This progress in rapid DNA analysis resulted in the production of increasingly automated instrumentation. Applied Biosystems™ RapidHIT™ ID System (ThermoFisher, Foster City, CA) is one of these two devices. With these new instruments the entire process of analysis takes around 90 minutes. In fact, the only manual step made by the analyst is the introduction of the sample in the instrument. After that all the other steps are automated; extraction amplification and capillary electrophoresis are integrated into the RapidHIT™ID system. The efficiency of the system has been investigated and demonstrated that is possible to obtain good quality profiles from saliva and blood samples (Gangano 2013).

Another instrument for rapid analysis that is widely used is the ANDE™ System (ANDE Corporation, Waltham, MA). Similar to the RapidHITsystem, ANDE is a fully automated system for STR analysis. In 2019 a multigroup study published the validation of the system using Flex Plex assay analyzing over 2000 buccal swabs. The results obtained from all the laboratories involved in the validation demonstrated the reliability and reproducibility of the analysis proving the robustness of the system (Carney 2019).

A group of experts known as the Rapid DNA task force was created by the FBI in 2018 to investigate the potential of the rapid DNA analysis for forensic evidence. In 2020, the task force, the DNA Working Group of the European Network of Forensic Science Institutes (ENFSI) and the Scientific Working Group of DNA Analysis Methods (SWGDAM), published a combined statement in *Forensic science International: Genetics*. They identified 5 main characteristics and goals that a Rapid DNA system must accomplish before it can be considered for forensic analysis and databasing (Hares 2020). Only the two instruments mentioned above, are currently approved by the FBI for use with the National

DNA Index System (NDIS) as a law enforcement booking station. However, research studies continue on Rapid DNA analysis. Recently promising results have been obtained on the analysis of touch DNA samples on cables and matchsticks (Martin 2022).

2.6.2. Direct PCR

Direct PCR is a technique largely used to avoid any pretreatment of the sample, reducing handling and contamination, as well as providing an analytical process that is less toxic, cheaper, and faster. Some research groups were able to amplify samples of whole blood without any extraction (Mercier 1990). In the late 1980s', a special sample retention paper (FTA) was developed to keep DNA stable while simultaneously allowing for ease of sample storage and use. As previously mentioned, the major advantage of this paper was that cell lysis and stabilization occur once the sample is added to the paper and that PCR amplification can be performed directly from paper punches without the need for further extraction. (Fowler 1988).

Several articles have been published using FTA paper as a direct method for PCR, some of them using just a buffer as pretreatment of the sample (Chomczynski 2006) others evaluating the direct amplification using the FTA compared to non-FTA methods (Hall 2014).

The main issue in performing direct PCR is the release of DNA from cells and the removal of interferences such as PCR inhibitors. In the past, there have been extensive studies on the development of novel PCR additives to improve amplification. Coextracted materials such as collagen, calcium, and humic acid affect PCR amplification and reduce yield

(Funes-Huacca 2011, Alaeddini 2012). Modified DNA polymerase enzymes, as well as enhancers in the PCR buffer such as BSA, have been developed to minimize the effect of PCR inhibitors (Panaccio 1993, Qu 2007, Bu 2008).

Direct PCR kits have been recently commercialized to be applied to forensic identification purposes, particularly in post-conviction testing, where the identity of the suspect is known (Vallone 2008). Validation of these commercial kits is critical. The results obtained by the different studies demonstrated that the use of these kits can produce reliable results when reference samples are analyzed, (Wang 2011), while for low template samples they provide recommendations for processing these kind of samples (Myers 2012).

2.6.3 Rapid-Direct PCR

Direct PCR can also be performed rapid. Numerous studies have been published that couple the two methodologies. In one of these studies, in 2002, the possibility of performing direct and fast PCR on buccal swabs was investigated for diagnostic purposes, using fluorescent probes and a high-speed thermal cycler. The results showed the possibility to genotype the samples in less than 30 minutes (French 2002).

In 2012 an interesting study was published, focused on the development of a direct and rapid protocol to analyze forensic samples coming from different sources as hair, blood, saliva, and semen. The study employed a specific DNA polymerase, the Phusion® Flash polymerase, and a fast thermal cycler system PIKO1 both from Bioké (Bioke´, Leiden, the Netherlands). The protocol allowed the amplification of forensic samples in less than 1 hour (Verheij 2012).

In 2013, a research study from Aboud demonstrated the ability to obtain a complete DNA profile from buccal swab in less than 25 minutes using the rapid polymerase z-Taq (Takara Bio, Clontech Laboratories, Inc., Mountain View, CA), AnyDirect F buffer mix (Bioquest, Korea) as a buffer, and Philisa Streck thermal cycler (Streck Technologies, New England, USA) (Aboud 2013).

Other experiments demonstrated that it is possible to generate a complete DNA profile of 7 STR loci with an amplification process under 7 minutes as shown in Figure 19 (Gibson-Daw 2018).

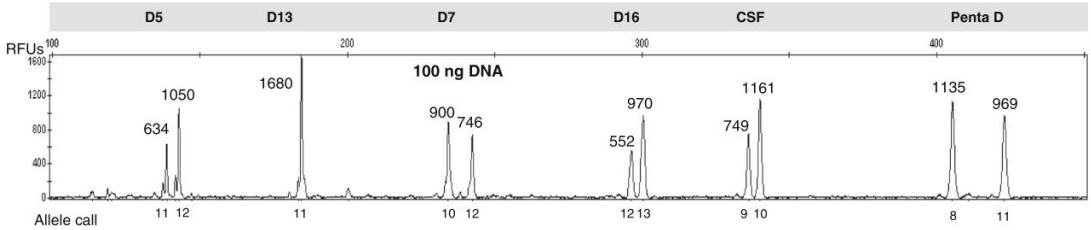

Fig.19 Electropherogram showing 100 ng of K562 control DNA standard. Results from 7 STR loci amplified using ultra-rapid PCR amplification (Gibson-Daw 2018)

The McCord group has continued these studies by developing an ultra-fast PCR protocol using direct amplification with a homemade multiplex of 9 miniSTR loci, a fast polymerase enzyme OmniTaq (DNA Polymerase Technology, Inc., Missouri, USA) and Philisa thermal cycler (Streck Technologies, New England, USA) (Boelens 2021).

Collectively, these results have provided the foundation for investigating methods to improve the DNA yield of forensic samples using a microwave oven as an extraction technique combined with a direct and rapid multiplexed PCR.

CHAPTER 3 Experimental Methods Microwave oven Calibration

3.1 Introduction

Since a goal of the project, was to develop a new extraction technique that would be low cost at first, we employed an Amazon Basics Microwave (700W that was $60) (Figure 20). Microwave energy imparted to samples will differ depending on the sample volume and position in the microwave. Too little energy may result in inefficient DNA recovery and too much energy may result in DNA degradation. Thus, calibration of the microwave oven is necessary before implementation. Three different types of microwave calibration tests were performed: temperature, retention and neon bulb excitation. For all of these experiments, the circular, rotating plate inside the microwave was removed so samples could be reproducibly placed in the same position during testing. During the first tests, locations to place the samples for conducting experiments were selected based on temperature, measured with a thermal couple and sample retention: observation of samples to remain in sealed tubes following microwaving. A rectangular plastic rack for biological samples was set in all possible locations inside the microwave (Figure 20).

Fig.20 Commercial Microwave oven used for preliminary calibration tests (left), and sample racks registration inside the oven.

3.2 Neon Bulb and sample retention test

3.2.1 Materials and methods

The neon bulbs represent a useful tool to detect the pattern of the microwave radiation since is not visible at the eye. The radiation is able to ionize the neon gas in the bulb, and when it is high enough the neon bulb glows. This is very helpful to identify the radiation pattern (Login 1998).

Next, individual neon bulbs NE-2 (OCSParts-Amazon) were inserted into 1.5ml tubes and then an entire 8 by 12 sample rack was filled with the tubes. The tubes with the neon bulbs were subjected to a continuous microwave exposure of 10s and the light emitted was observed and recorded. Figure 21 show the arrangement of the neon bulbs in the tubes.

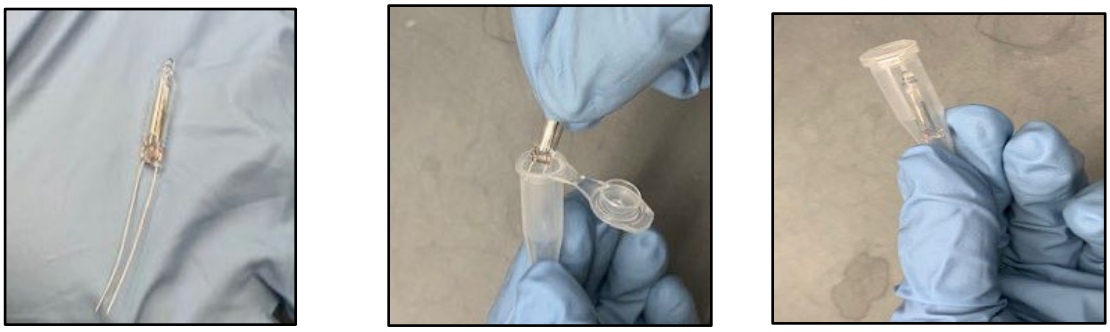

Fig.21 Neon bulbs set-up. First the electrodes were cut to fit the tube length, then they were carefully placed in the tube avoiding any contact between the filaments.

This experiment was performed to further check, which bulbs turned on and, which did not, to demonstrate differences in the microwave energy.

Additionally, we measured the retention time of the samples and the condensation created when exposed to the microwaves. The retention time is defined as how long a tube with a sample can tolerate heat in the microwave before the tube opens, due to increased pressure. The condensation of liquid observed on the inside of the tops and sides of the tubes,

indicates where hot and cold spots are located in the microwave. To detect this effect, 100µl of water was spiked into 1.5ml and 0.5µl tubes, placed in all positions in the rack, as showed in Figure 22, and then exposed to the microwave for several cycles: 8 s, 16 s, 24 s, and 30 s with a pause of 10s between each cycle.

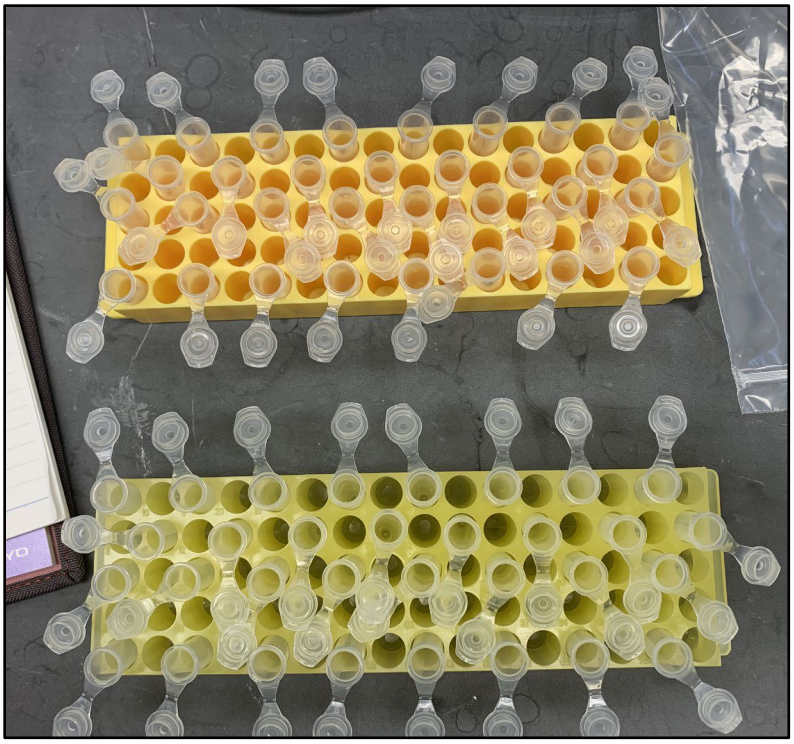

Fig.22 Arrangement of 1.5 ml tubes in the sample racks. The tubes were filled with 100µl of water and exposed to several cycles of microwaves.

3.2.2 Results and Discussion

The neon bulb firing (Figure 23), and water condensation tests were employed to determine microwave energy and distribution in the chamber.

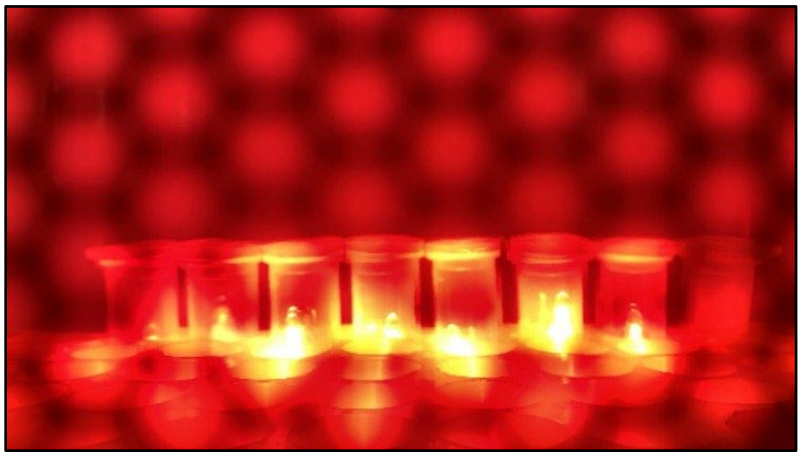

Fig.23 Picture taken while neon bulbs were exposed to the microwaves. It is possible to visualize the different lighting pattern create by the radiation.

In Figure 24 four locations were selected for the preliminary tests. The four locations were chosen among those in which we observed medium level of condensation in the tube after 30 seconds of microwave exposure. The medium level of condensation was indicated by the presence of some condensation on sides of the tube but not saturation (Figure 25).

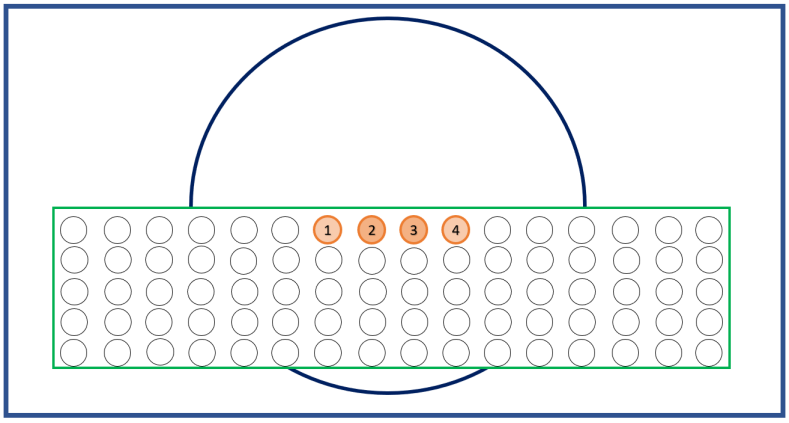

Fig.24 Location selected. The preliminary results in Microwave oven calibration allowed the selection of 4 preferable locations for the samples.

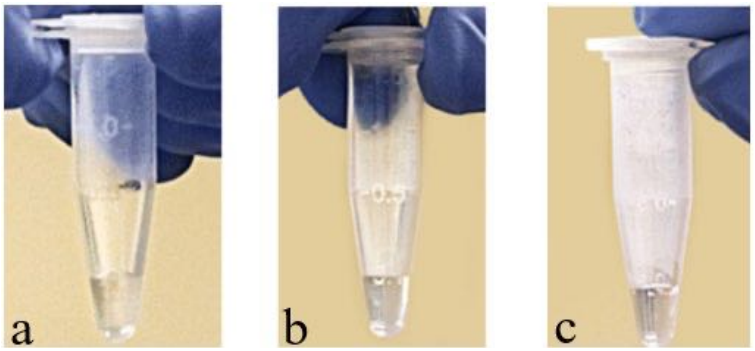

Fig.25 Example of different status of condensation observed after microwave oven exposure. a=Lower level of condensation, b= Medium level of condensation, c= High level of condensation

The observations were made empirically since the off-the-shelf microwave does not provide any temperature monitoring nor energy measurements delivered to each sample, a limitation of the Amazon and other off the shelf microwaves. These features were also tested in experiments using a computer-driven Microwave PELCO Biowave Pro + (Ted Pella Inc. Redding, CA) capable of measuring energy and temperature, to permit a comparison of the results with the commercial oven and its effect on the temperature changes that occur during heating, using the software connected to the computer-driven instrument.

3.3 Temperature tests

3.3.1 Materials and Methods

Temperature tests were performed on 4 selected locations. A stainless-steel thermocouple probe connected to a digital thermometer was used. To check if the volume and location could affect the temperature in the tube, the temperature was measured at the 4 locations using different volumes of water: 30µl, 50µl,75ul, and 100µl. Additionally, a water sample of 30µl was heated in the microwave for 8sx3, 12sx3, 16sx3, 20sx3, 30sx3 (with pauses of 10s between each microwave singular cycle) and for 24s with no pause. A temperature probe was inserted after each cycle in each tube to evaluate the impact on the temperature increase inside the sample at different volumes and time duration. The experiments were performed one sample at time with periods of resting until the internal temperature measured with the probe returned to room temperature.

3.3.2 Results and Discussion

Figure 26 shows the results of the temperature measured at each of the 4 locations previously selected, following 3 steps of heating using 8s with an interval of 10s rest between each cycle. The samples were 0.5µl tubes filled with different volumes of water. The results indicate that location number 4 is heated less for all different volumes. Location number 1 on the other hand reached higher temperatures after the exposure, unrelated to the volume, except for 30µl sample. In terms of volume, a sample of 100µl seems to have high variability when put in different locations. Therefore, based on the microwave cycle selected, since we want to avoid the hot and cold spots, locations 2 and 3 were selected for the experiments.

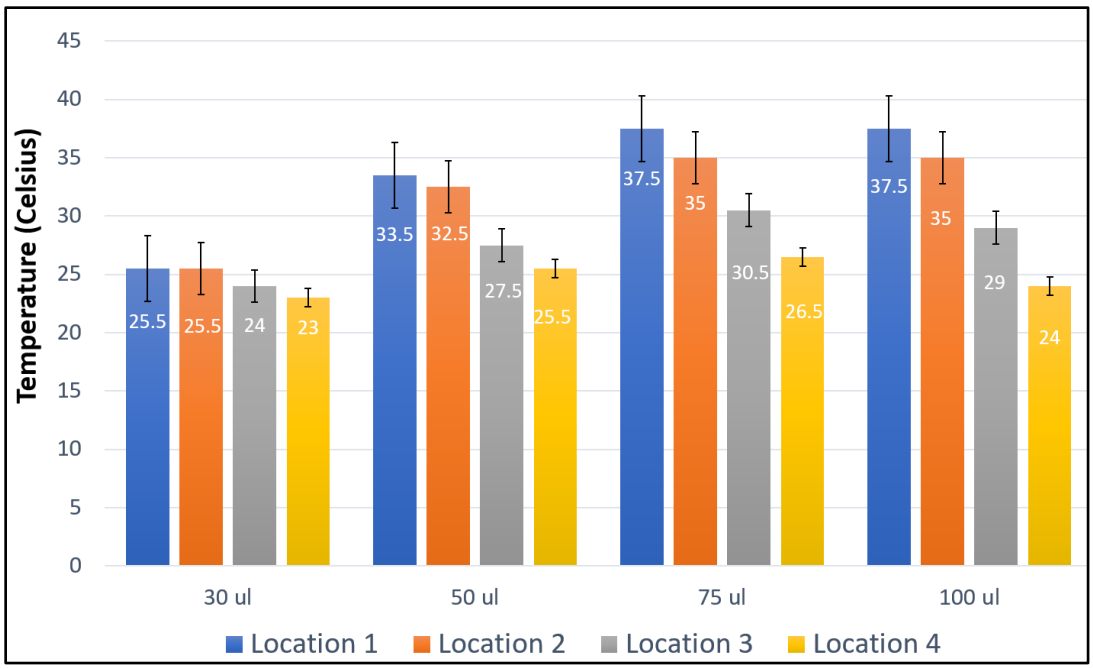

Fig.26 Relation between temperature and volume in the selected location, standard error bars are shown.

Next, we analyzed a 30µl volume sample at different microwave cycles and in all 4 locations. In Figure 27 results are shown. Higher variability in terms of temperature is observed for 20s x3 cycling and 24s cycling. Ultimately the best temperature (reached using different microwave parameters) is that which can be correlated with cell membrane disruption for optimal release of the nucleic acids. In an automatic extraction technique, the samples are incubated with proteinase K for 15–20 minutes at 56°C, a temperature that a commercial microwave of 700 W cannot reach unless the sample is microwaved for more than 5 minutes. This process would result in tube opening or melting.

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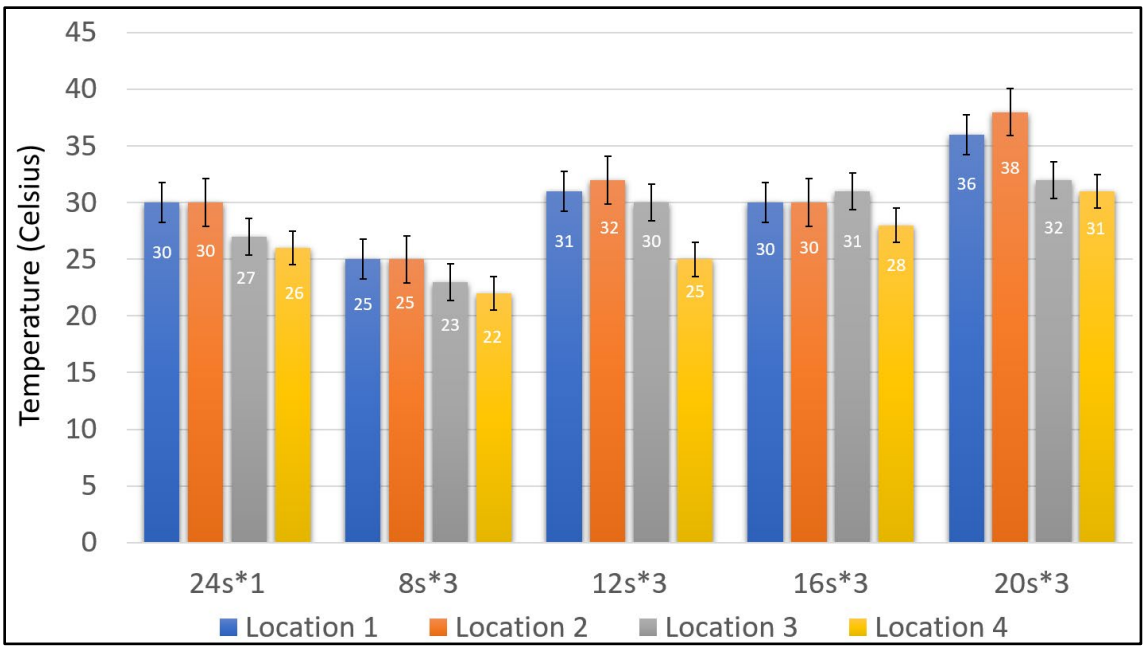


Fig.27 Relation between temperature and different microwave heating cycles, standard error bars are shown.

The results obtained by the preliminary tests on the commercial microwave oven were used to calibrate the computer-driven microwave and optimize the sample analysis.

Further optimization using DNA yield and PCR STR results will be discussed in the next sections.

CHAPTER 4. Conductivity

4.1 Introduction

Saliva samples were used to determine the effect of salt on the relative amount of microwave energy imparted to the sample. Conductivity tests were performed before microwave treatment, using a Horiba B-173 Twin Cond Conductivity Meter, showed in Figure 28. This instrument is the size of a pen and contains a small sensor cell at the tip, where the drop of the sample is placed, and the digital display shows the results.

Fig.28 Conductivity meter Horiba B-173 Twin Cond

4.2 Materials and methods

A saliva sample was diluted with three different buffers d.i.H_20, TE, and tween-20. The dilutions used were as follows: saliva 100%, diluted 75%, diluted 50%, diluted 25%, diluted 10%, and only buffers. For each buffer dilution, three measurements were taken.

4.3 Results and Discussion

Figure 29 shows the results of the conductivity test. Unfortunately, the high salt concentration is a strong inhibitor of PCR, therefore it is crucial to keep this concentration low. However, the dilution of the saliva can reduce ionic strength. The results demonstrated that the conductivity decreased linearly with saliva sample and buffer dilutions. The results suggest avoiding the use of whole saliva 100% to decrease any chance of inhibition of the sample. Based on these results, water or tween-20 can be used to dilute the samples to effectively reduce the salt content and conductivity in the saliva samples.

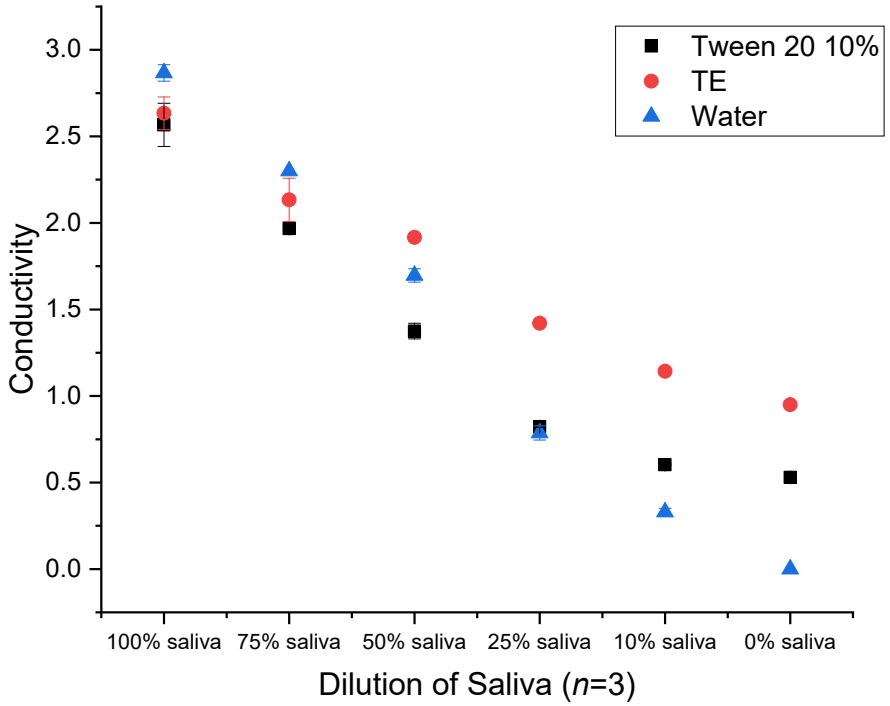


Fig.29 Conductivity test results. Three buffers, Tween-20, TE and Water, were used to dilute a saliva sample using the following dilution scheme: saliva not diluted, diluted 75%, 50%, 25%, 10% and only buffer. Once diluted, conductivity measures were taken. Each sample was measured 3 times. Errors bars are shown.

CHAPTER 5: Quantification

5.1 Introduction

In these preliminary tests, we wanted to use quantification as a further tool for our analysis. DNA yield was evaluated from whole saliva, saliva cellular pellets, or supernatant following centrifugation.

After determining the sample type that provided the highest DNA yield, testing was performed on varying microwave energy and duration to optimize yields.

Real-time PCR represents an amplification step that coupled with the use of dyes can determine the concentration of the DNA in analysis (Higuchi1993).

In forensic analysis, quantification is a mandatory step. Crude samples like saliva may contain inhibitors and bacterial enzymatic activity that may degrade DNA (Opel 2010).

To increase specificity in the quantification step, ALU primers were used. These particular sequences are present in hundreds of thousands of copies in the human genome, and therefore a perfect marker for detecting human DNA (Nicklas 2003, Mighell 1997). One forward primer and one reverse primer of ALU employed amplifies a short fragment of 82 bp. The use of a second reverse primer results in amplification of a longer fragment of 201 bp of DNA that may be helpful since the differential detection of the longer fragment could provide insight into whether a sample is degraded and thereby provide a more accurate interpretation of results.

5.2 Materials and methods

5.2.1 Sample collection and microwave extraction

Five saliva samples were analyzed (4 females, 1 male). Sample collection was approved by the institutional review board at Florida International University (IRB-19-0354). Saliva samples were collected by spitting into 1.5 ml tubes or by collecting buccal cell/ cheek swabs, rubbing for 10 sec the inner part of each cheek. The samples were then stored at 4°C right after collection to avoid bacterial growth. Whole saliva samples, when needed, were taken from the fridge (not more than 1 hour following storage) and aliquoted into other tubes. The buccal swabs were cut to fit into a 1.5ml tube with a spin basket on top. Labeling tape was used to seal the lid to avoid any loss. Samples were centrifuged at 13000 rpm for 2 min, the spin basket with the swab was then removed, and the DNA containing cellular fraction was collected after centrifugation from the bottom of the tube. The sample was then split into two aliquots and one aliquot was stored momentarily (no more than 15 minutes) in the fridge.

The samples were exposed to microwaves three times for 8s with a 10s pause between each cycle, based on the work of (Giberson 1995). Additionally, replicate samples were subjected to 24s of continuous microwaving.

5.2.2 Real-Time PCR and Fluorometric quantitation

In the quantification step, two instruments were used. One method employed a Qiagen Roto-Gene 6000 instrument (Qiagen, Inc., Valencia, CA) The other method, used to quantify extracted samples was the Qubit4 a fluorometer instrument from Invitrogen (Waltham, MA). The qPCR used for the Qiagen Roto-Gene instrument was a modified Alu

PCR (Opel 2008). The kit used for the extracted sample was Qubit 1X dsDNA HS (High-Sensitivity) Assay Kit (Invitrogen Waltham, MA).

An automated liquid handler CORBETT Robotics CAS1200 was used for the direct PCR of samples to prepare the dilutions, the master mixes, and the real-time samples before the analyses. The samples were then quantified using a Rotor-gene SYBR green PCR kit from Qiagen (Qiagen, Inc., Valencia, CA). SYBR green is an intercalating dye that binds the dsDNA; therefore, it is possible to determine the amount of DNA analyzed. The ALU primers, with an amplicon length of 82 bp (Opel 2008), were bought from Integrated DNA Technologies Inc. IDT, (Coralville, IA).

The primer sequences were the following:

ALU-F 5'GTCAGGAGATCGAGACCATCCC 3'

ALU-R 5' CCACTACGCCCGGCTAATTT 3'

The examined samples contained 2µl of a saliva sample, 0.2µl of Alu-F, 0.2µl Alu-R, 12.5µl of SYBR-Green Master mix and 10.1 µl of water for a final volume of 25µl.

A known standard DNA was serially diluted to generate a standard quantification calibration curve. The real-time reaction, therefore, included eight known standard DNA dilutions (4 ng/µl, 2 ng/µl, 1 ng/µl, 0.5 ng/µl, 0.25 ng/µl, 0.125 ng/µl, 0.0625 ng/µl, and 0.03125 ng/µl), a blank sample, and the unknown diluted saliva, pellet and/or supernatant samples. The PCR protocol used consists of an initial activation step of 5 min at 95°C, and two-step cycling repeated 40 times: 5s at 95°C, and 10s 60°C. The total run time is about an hour.

Initially, we analyzed the different matrices of saliva, whole saliva, pellet, and supernatant. Quantification was performed the same day on the same sample, microwaved and not microwaved, in duplicate and in replicate for three days.

To understand if different buffers could also affect the quantification, a saliva sample was diluted at 100%, 90%, 75%, 50%, 25%, and 10% with di H20, TE, and Tween-20. For each dilution/buffer sample, 3 replicates were made and quantified.

Moreover, we tested the variability in the amount of DNA recovered after the collection of a whole saliva sample from the same donor in a period of 5 days. Each day 3 replicates of the saliva sample were diluted 75% with water and then quantified.

5.3 Results-Discussion

Quantification of the amount of DNA in different saliva substrates was performed. In this step, the goal was to determine which saliva collection and processing method would yield the highest DNA recovery and most complete STR amplification results after microwave extraction. Three different matrices were collected from the same saliva sample: whole saliva, supernatants, and pellet. Each of the three sample types was analyzed in duplicate, and the quantification experiment was repeated for 3 days with the same protocol. The pellet with or without microwave treatment provided the highest yield compared to the other two sources. Whole saliva is easier to process as centrifugation to collect pellets, may be difficult to use in the field.

In the quantification results, variability between samples was observed on different days, likely due in part to the intricate nature of saliva in which bacterial growth, and the varying content of protein and salts can differentially inhibit the analytical processes resulting in

differences in quantification results. For example, in the supernatant experiment, the samples that were not microwaved resulted in slightly higher DNA yields vs microwaved samples and therefore should be further investigated.

Variability in the recovery of DNA in the whole saliva from the same donor before any microwave exposure was also observed. To test this issue, whole saliva was diluted 75% with water, collected each day for 5 days, and 3 replicates for each day were quantified with real-time PCR. As shown by Figure 30 there is variability between days even if the sample was collected from the same person.

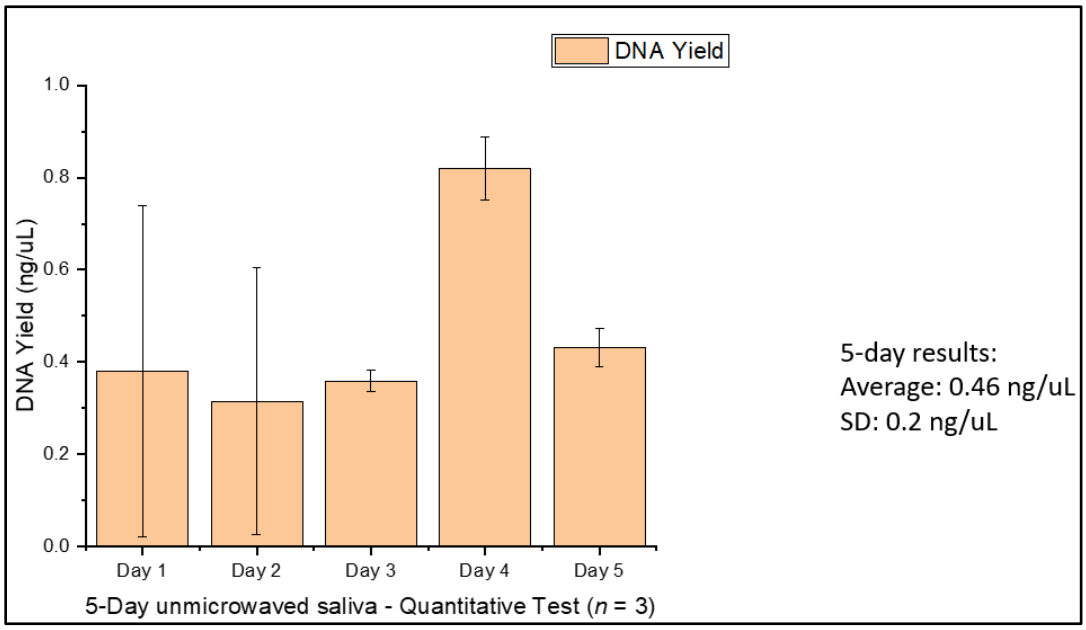


Fig.30 Quantification test on same donor saliva. A saliva sample, from the same donor, was collected, diluted 75% and quantified (3 replicates) each day in a period of 5 days. Standard deviation bars are shown.

Quantification of diluted saliva with the three buffers used in the conductivity tests was performed, to assess the impact on yields in different dilutions. The results shown in Figure 31 indicated that 90%and 75% dilutions provided comparable results to 100% whole saliva.

Results also indicate that water provided yields as good or better than TE and tween-20. This result is particularly interesting since we hypothesized that TE or Tween-20 might improve the yield of the samples over water as TE may stabilize the DNA from nuclease activity and tween-20 might help release nucleic acids from cells.

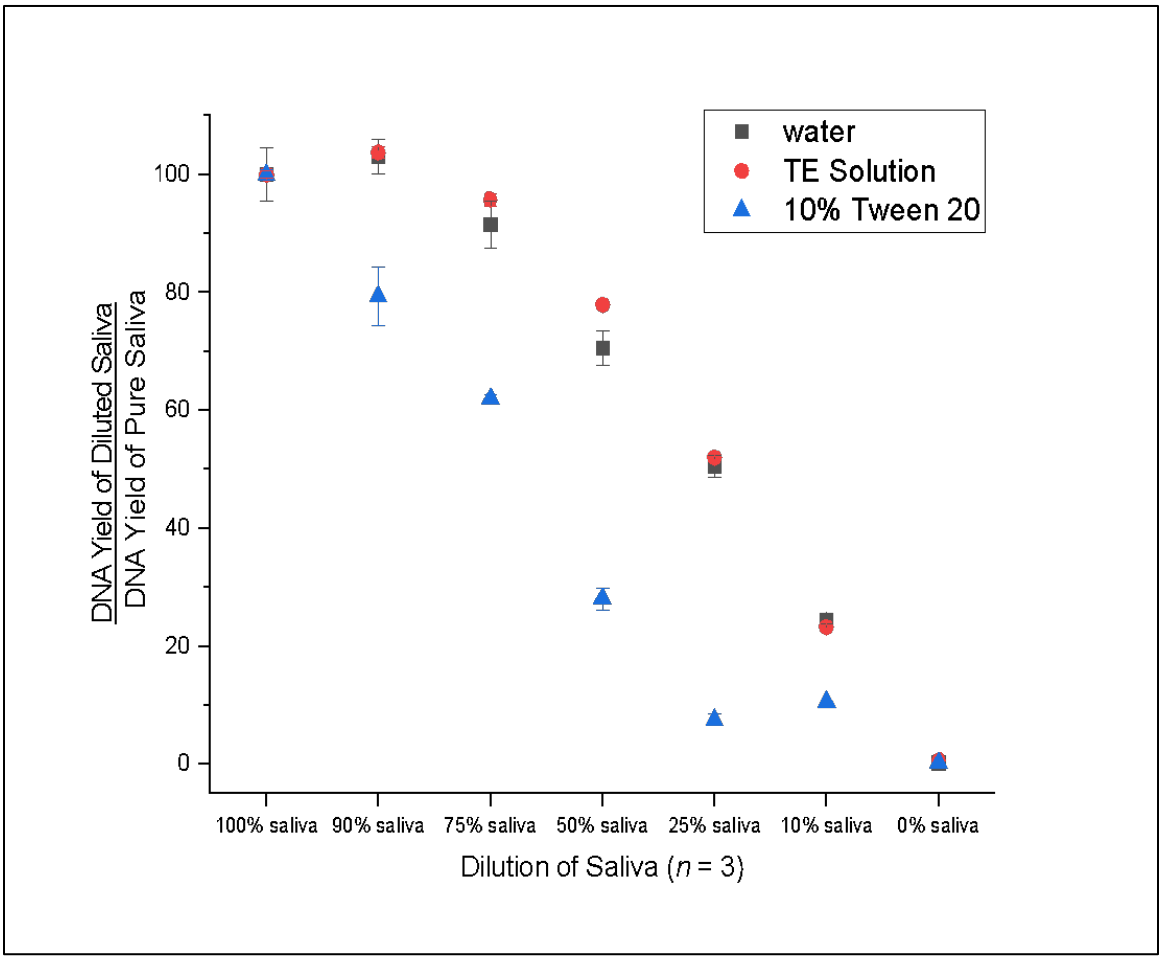


Fig.31 Results of quantification tests on diluted saliva with water, TE, and Tween-20. A saliva sample was diluted using the three different buffers at several percentage of dilution: saliva not diluted, diluted 75%, 50%, 25%, 10% and only buffer. The samples were then quantified in replicates of 3 for each buffer and dilution fraction.

5.4 Optimization of computer-driven microwave

To establish the time and power required to efficiently analyzed the samples, tests on Saliva samples were performed on the computer-driven microwave.

In the first studies 3 replicates of a saliva sample coming from the same donor were exposed at the highest energy power of 700W for different times: no microwave, 10 sec, 20 sec, 30 sec, 40 sec and 50 sec. They were later quantified using Real time PCR (protocol cited above).

Figure 32 shows the results of the first test. No microwave exposure, and 40 seconds resulted in the highest yields.

In the second test, using 40 sec as Microwave exposure time, another set of saliva samples replicates, coming from the same donor, were exposed to different range of power: no microwave, 300W, 400W, 500W, 600W and 700W. The samples, once microwaved, were quantified. The results demonstrated that 300W of power gave the highest DNA yield in the experiment (Figure 33).

The goal of the last test was to check the yield of DNA, using the power and time settings of the Microwave oven determined in the first studies. Therefore, 3 replicates of saliva samples coming from the same donor, were exposed 40 sec to the microwaves at the following energy levels: no microwave, 100W, 150W, 200W and 300W. After the microwave treatment they were quantified. DNA yield variability was observed for replicates of saliva samples that may be due to heterogeneity in the original saliva samples (Figure 34). The DNA yield results support the use of 40 seconds at 300W.

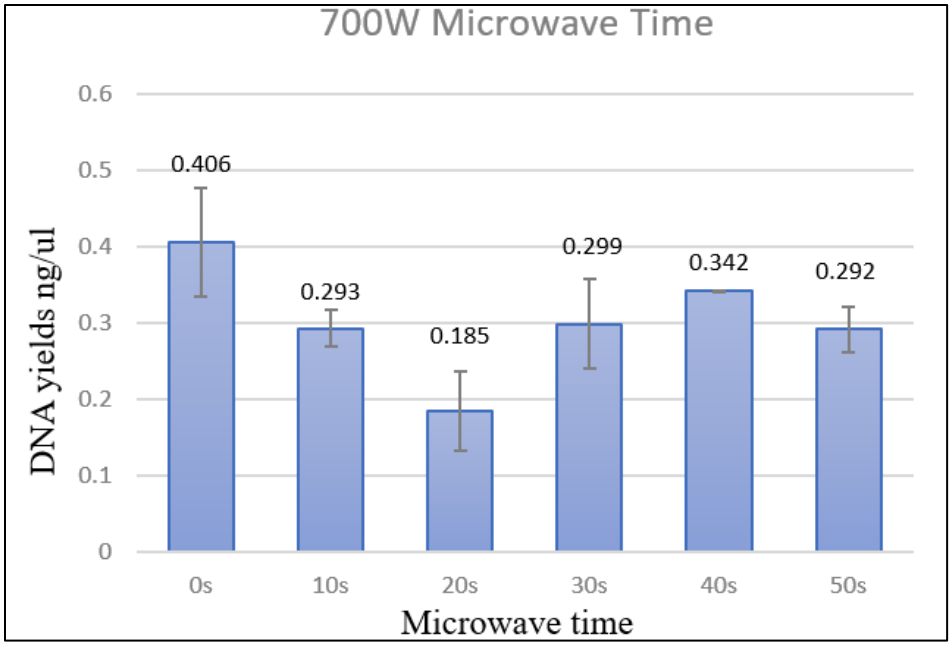


Fig.32 Optimization computer driven microwave. Test 1. Saliva samples were microwaved in triplicates for 0s, 10s, 20s, 30s, 40s and 50s at the power of 700W. Once microwaved they were quantified. Error bars are shown

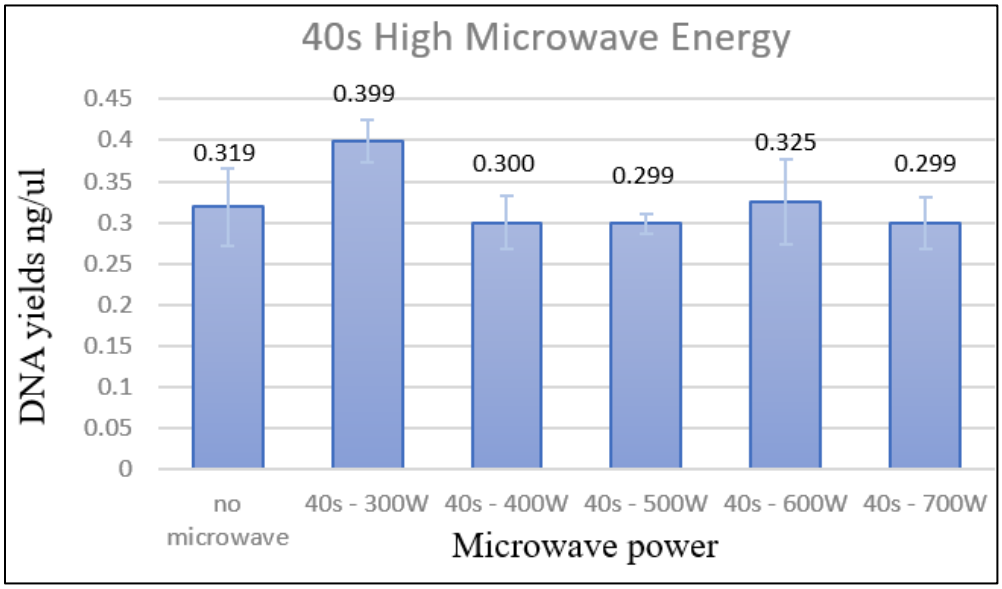


Fig.33 Optimization computer driven microwave. Test 2. Saliva samples were microwaved in triplicate for 40 sec at different energy levels: no microwave, 300W, 400W, 500W, 600W and 700W

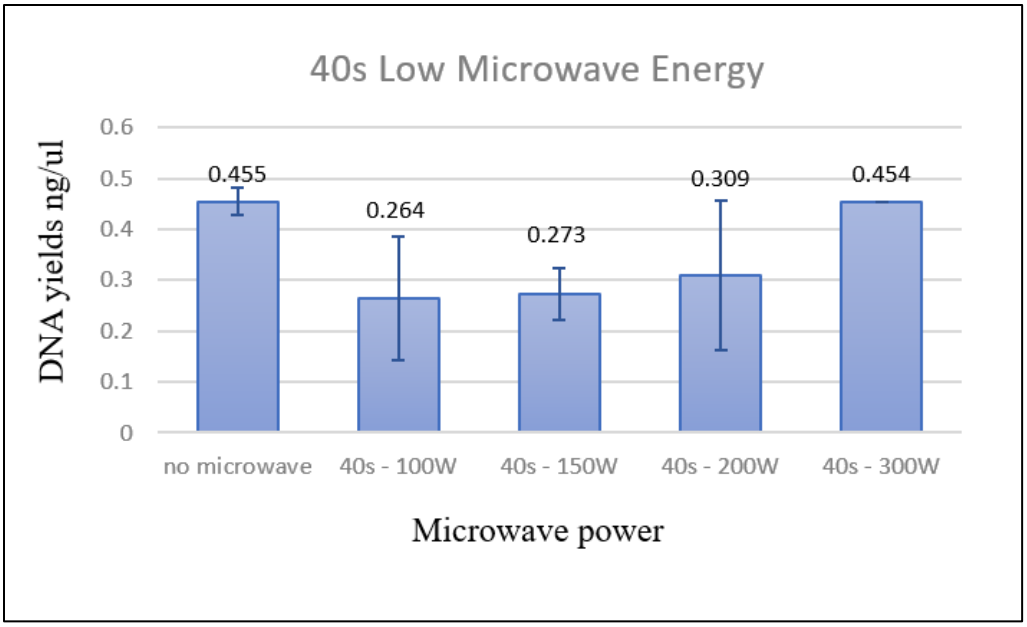


Fig.34 Optimization computer driven microwave. Test 3. Saliva samples were microwaved in triplicate for 40 sec at lower energy levels: no microwave, 100W, 150W, 200W, and 300W
.

CHAPTER 6: Amplification and Genotyping

6.1 Introduction

The amplification process is a critical step in forensic DNA analysis especially when developing a new protocol with a homemade PCR multiplex. Developing a new multiplex requires the testing and evaluation of many parameters. Furthermore, in this project direct PCR of samples not subjected to a common extraction and purification technique for rapid amplification protocols is required since the microwave extraction does not include any purification steps. Therefore, the microwave isolated DNA samples can be affected by any intrinsic contaminants or inhibitors contained in the tested samples.

In this study, the amplification process can be divided into two phases.

In the first step to assess the primer function and whether the multiplex protocol previously developed was functioning, a positive control sample, 2800M (Promega Corporation Madison, WI) was amplified. After further optimization, the protocol was then applied to microwave extracted DNA from all three body fluids. In the results section a representative profile obtained during protocol development and modification are shown.

6.2 Materials and methods

6.2.1 Direct Polymerase

The DNA polymerase employed for the fast and direct amplification is OmniTaq (DNA Polymerase Technology Inc, Saint Louis, MO). This polymerase is a mutant of the common *Taq* polymerase, and it's commonly used for trace samples and samples deposited on difficult substrates such as soil or samples inhibited by contamination with humic acid,

tannins, or urine. This polymerase was shown to be efficient in direct analysis (Kermekchiev 2012).

It shows particular sensitivity even when used with old, dried blood samples collected in a dusty location (Kermekchiev 2012). The manufacturers recommend coupling OmniTaq with a buffer containing a PCR enhancer cocktail (PEC-1) to improve the sensitivity and DNA yield. This additive also assists in the amplification of crude samples (Zhang 2010). It contains a high salt, and D-(+)-Trehalose that enables thermostable enzymes to maintain their normal activity (thermostabilization) and may also results in maintenance of polymerase activity at high temperatures (Spiess, 2004). This compound, however, is known as an effective osmoprotectant and should enhance the PCR reaction in a fashion similar to that of betaine and proline (Zhang 2010).

All the samples were amplified using a fast thermal cycler, Philisa® from Streck Biosciences company. The characteristics of this thermal cycler make it a very useful tool for rapid PCR reactions and on-site field analyses. Compared to common thermal cyclers, the Philisa® is compact and light. It has a high heating ramp rate (up to15c/s) and a rapid cooling ramp (rates up to 12c/s), which permits rapid PCR protocols. This characteristic is mainly due to peculiar thin-walled tubes specifically designed for the instrument, which provides an efficient heat transfer due to an increased surface-to-volume ratio (Figure 35). Analysis of up to 8 samples at a time in less than 15 minutes can be achieved.

Fig. 35. Fast Thermal cycler Philisa® from Streck Biosciences. Details of the heat block and the design of the tubes are shown.

6.2.2 Samples collection and Microwave extraction

In the first set of experiments, positive control 2800M (#DD7101) from Promega was purchased. This positive control was used to develop the amplification protocol. The allelic Ladder 24 plex produced by Qiagen was employed and amplified with our amplification protocol to create the allelic ladder used to size and genotype our samples.

The methods used for collection of the body fluids consisted of the following steps: Saliva samples were collected by having volunteers spit into 1.5ml tubes. The samples were then diluted to 1% using deionized water, creating a stock solution of 200µl final volume (2µl saliva+198µl water). 10µl of this solution was exposed to 20 seconds of microwaves at 300 W of power.

Blood samples (drops) were collected using a finger prick in 500ul tubes. The samples were then diluted 1% using water, (2µl blood+198µl water) creating a stock solution of 200ul final volume. 10µl of this solution was exposed to 40 seconds of microwaves at 300 W of power.

Semen samples were collected in 50 ml tubes. The samples were then diluted 0.5% using water, (2µl semen+398µl water) creating a stock solution of 400ul final volume. 10µl of this solution was exposed to 40 seconds of microwaves at 300 W of power.

Mixture samples (saliva and semen) were produced using two individual samples prepared according to the protocols above. The mixture was then created in several ratios. For example, in the mixture ratio saliva: semen 1:10, at 10µl of the final volume, 1µl of saliva and 9µl of semen were used to create the mixture. The sample solution of 10µl was then subjected to microwaves for 40 sec at 300 W. Additional ratios of 1:1 and 10:1 were also produced. Sample collection was approved by the institutional review board at Florida International University. (IRB-19-0354)

6.2.2 PCR amplification

The 11 mini STR loci used in this project were previously developed (Boelens 2021). The primers were designed with different fluorescent dyes on the 5'of the forward primer. All forward and reverse primers (D2S1338, D2S441, D7S820, D8S1179, D10S1248, D18S51, D21S11, Amel, FGA, TH01, and vWA) were obtained from Thermo Fisher except for the vWA reverse primer which was purchased from Integrated DNA Technologies Inc. IDT, (Coralville, IA) and had a different structure. Due to overlap that may occur between TH01 and vWA, the reverse primer of vWA was designed to have a 5' end spacer, ISP 18, an 18-atom hexa-ethylene glycol. The spacer used in this analysis is a synthetic and physical long arm that allow the fragment to be longer, slowing down its electrophoretic migration, therefore avoiding the overlap between two tandem repeat amplicons TH01 and vWA (Table 1).

The primer mix including all the miniSTRs primers amounts and concentrations were previously determined by Dr. Mariot in our lab (Boelens 2021). In this study, the initial primer amounts were modified throughout the experiments, to optimize the results for specificity, yield, and sensitivity.

D2S1338	90-142	F	TGGAAACAGAAATGGCTTGG	rox/RED
		R	GATTGCAGGAGGGAAGGAAG	
D21S11	153-211	F	ATTCCCCAAGTGAATTGC	
		R	GGTAGATAGACTGGATAGATAGACGA	
D10S1248	79-123	F	TTAATGAATTGAACAAATGAGTGAG	joe/GREEN
		R	GCAACTCTGGTTGTATTGTCTTCAT	
FGA	125-281	F	AAATAAAATTAGGCATATTTACAAGC	
		R	GCTGAGTGATTTGTCTGTAATT	
D8S1179	86-134	F	TTTGTATTTCATGTGTACATTCGTATC	tamra/YELLOW
		R	ACCTATCCTGTAGATTATTTTCACTGTG	
D7S820	136-176	F	GAACACTTGTCATAGTTTAGAACGAAC	
		R	GTTTCTTTCATTGACAGAATTGCACCA	
D2S441	78-110	F	CTGTGGCTCATCTATGAAAACTT	FL/BLUE
		R	GAAGTGGCTGTGGTGTTATGAT	
D18S51	123-203	F	TGAGTGACAAATTGAGACCTT	
		R	GTGTGGAGATGTCTTACAATAACAGT	
Amel	212-218	F	ACCTCATCCTGGGCACCCTGG	tamra/YELLOW
		R	AGGCTTGAGGCCAACCATCAG	
TH01	51-98	F	CCTGTTCCTCCCTTATTTCCC	Purple
		R	GTTTCTTGGGAACACAGACTCCATGGTG	
vWA	88-142	F	AATAATCAGTATGTGACTTGGATTGA	
	(104-158)	R*	ATAGGATGGATGGATAGATGGA	

Table 1. List of STRs, primers' sequences and relative fluorescence assigned

To assess the performance of the primer sets and amplify an allelic ladder for the allele call, standard PCR amplification of extracted DNA samples and allelic ladders was performed. AmpliTaq Gold (Thermo Fisher) and Cesium Klentaq (DNA Polymerase Technology Inc, Saint Louis, MO) were the selected enzyme used as recommended by the manufacturer's protocol.

After many attempts, we realized that TH01 locus occupies a position close to the beginning of the electrophoretic run and therefore, is hard to detect (Fig. BBBB)

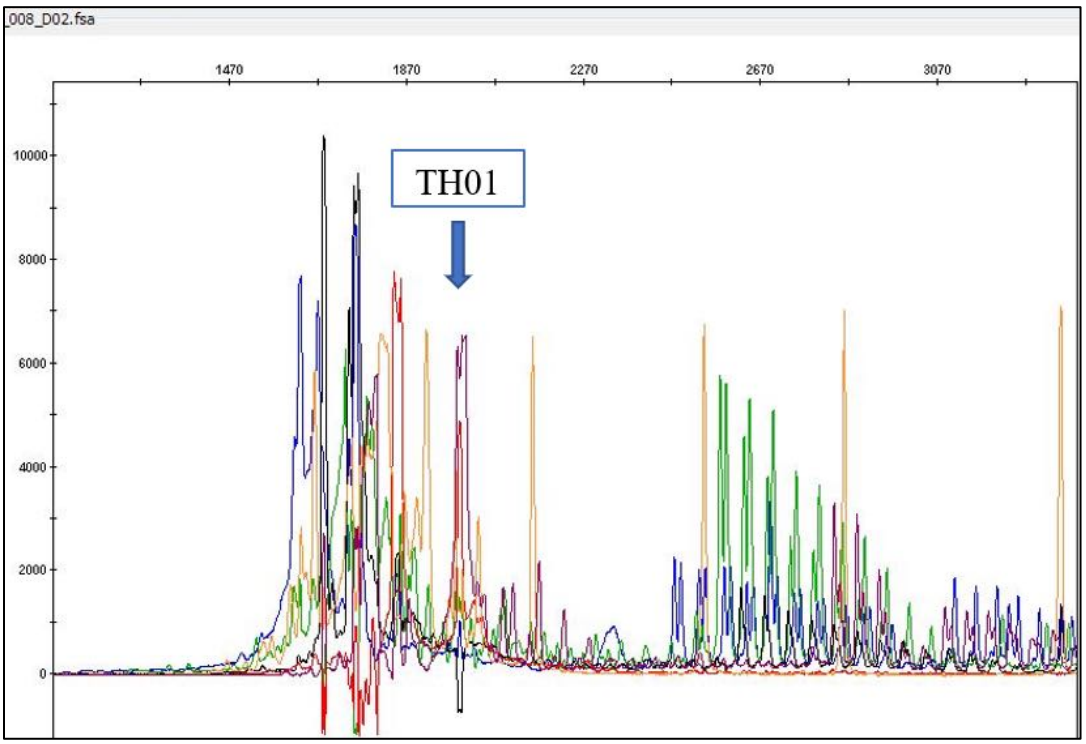


Fig.36 Allelic Ladder capillary electrophoresis, raw data. The starting point of TH01 is indicated by the arrow. The raw data shows the primer peak overlapping with the peak of TH01. Instrument used, ABI 3130XL.

As part of developing the method also D2S1338 was initially removed from the experiments and added again in the mixture study. The primer mix was prepared according to the following protocol (Table 2).

Each volume amount is doubled to include Forward and Reverse Primer

STR LOCI	Primer Concentration	Volume in Primer Mix
D2S441	0.23µM	1.3µl
D7S820	0.40µM	2.1µl
D8S1179	0.54µM	2.7µl
D10S1248	0.47µM	2.5µl
D18S51	0.30µM	1.6µl
D21S11	0.28µM	1.5µl
Amelogenin	0.27µM	1.4µl
FGA	0.34µM	1.8µl
vWA	0.42µM	2.2µl

Table 2. Primer's list, concentrations, and amount used to create the primer mix. The total volume of the primer mix created was 38µl, from this total volume we used 3.6µl for the PCR mix.

Sample input	Buffer 10X Omnitaq	dNTPs 2.5 mM	Primer Mix	PEC 2X	BSA 20mg/mL	$MgCl_2$ 25 mM	OmniTaq
2µl	1µl	1µl	3.6µl	0.4µl	0.1µl	0.5µl	0.6µl

Table 3. Master mix direct/fast PCR amount per sample, final volume 10µl

All the microwaved and not microwaved samples were amplified in the Philisa fast thermal cycler using the following PCR protocol in Table 3.

Initially, several conditions were tested including hot start duration, primer concentrations, and cycling parameters. The cycling condition is one of the parameters that was optimized. At first, the initial testing using the cycling protocol developed by Boelens failed, therefore two other cycling protocols were tested

Cycling conditions				
	Denaturation	Annealing	Elongation	N. cycles
A	98ºC x 2 sec	62ºC x 7 sec	-	32
B	94ºCx 90sec	94ºCx 2sec	62ºC x 20 sec	30
C	94ºCx 60sec	94ºCx 2sec	62ºC x 20 sec	30

Table 4. PCR cycling conditions optimization performed using Philisa Streck Thermal cycler. Three different protocols were used; A, B, and C.

POSITIVE CONTROL 2800M -1ng/µl									
STR LOCI / CYCLING CONDITIONS	**D2S441**	**D18S51**	**D10S1248**	**FGA**	**D8S1179**	**D7S820**	**AMEL**	**D21S11**	**vWA**
A	83-109	50-DO	DO	120-124	DO	DO	DO	173-119	DO
B	4419-4017	4875-3952	6943-7381	6676-6824	566-550	3160-3018	2923-3662	8069-8442	5174-3710
C	3573-3430	4598-4750	7382-7247	6666-6898	291-355	2945-3175	1482- 1643	8187-8460	4522-3978

Table 5. Results obtained from the amplification and genotyping of positive control 2800M 1ng/µl using three different protocols: A, B, and C (Table 4). Peak heights are shown in terms of the relative unit of fluorescence (RFU)

The results show that protocol B yielded amplicons for all loci, with higher peak heights compared to the other two protocols. Based on these results obtained, protocol B was selected for downstream testing.

The positive control was later amplified at different concentrations to confirm the ability of the amplification protocol to obtain a complete profile at a lower DNA input amount (see Table 6 below).

STR LOCI - PEAK HEIGHTS (RFU)									
	D2S441	D18S51	D10S1248	FGA	D8S1179	D7S820	AMEL	D21S11	vWA
2800M_1ng/µl	4419-4017	4875-3952	6943-7381	6676-6824	566-550	3160-3018	2923-3662	8069-8442	5174-3710
2800M_0.5ng/µl	1836-1939	1893-2188	4252-3564	3273-4374	181-136	1224-1225	1161-1356	4439-4137	2256-1708
2800M_0.25ng/µl	746-774	1373-1155	2874-2210	3861-4140	121-75	858-880	742-680	2877-2889	1645-1885
2800M_0.125ng/µl	422-363	277-170	574-864	724-239	DO-65	154-224	269-128	177-143	204-201

Table 6. Results obtained from the amplification and genotyping of positive control 2800M 1ng/µl, 0.5ng/µl, 0.25ng/µl and 0.125ng/µl using B protocol. Peak heights are shown in terms of the relative unit of fluorescence (RFU)

6.2.3 Genotyping

One of the methods used to create DNA profiles (DNA typing) is the Capillary gel Electrophoresis (CE).

Capillary electrophoresis is an analytical technique that allows the separation of any charged element, in this case, nucleic acid, that migrates from one pole to another when an electric voltage is applied (Li 1992).

CE consists of three main steps: injection, separation, and detection. After the amplification, the PCR products need to be denaturated. To be analyzed by the CE, the DNA has to be in a single stranded form. First, a master mix is prepared with a specific amount of Hi-Di™ formamide (Applied Biosystems®) that denatures the DNA and an Internal Lane Standard (ILS) to determine the fragment size. The mixture created is delivered to the sample tray. The amplified DNA is then added to the mixture in the sample tray. The samples are ready to be injected into the capillary.

The capillary and the electrode are immersed in the buffer until the oven reaches the target temperature.

As the instrument reaches the temperature set by the operator the capillary full of polymer moves together with the electrode toward the sample tubes.

As the voltage is applied the DNA fragment charged negatively are injected into the capillary and start their migration to the opposite positive pole. The polymer allows the separation by size; therefore, the larger fragment will be the slowest. Moreover, the polymer is used to control the Electroosmotic flow (EOF).

This phenomenon is caused by the charges of the inner wall of the capillary. The negative charges attract the cation in the buffer, their interaction creates an electrical double layer. This flow can affect the efficiency and resolution since there is no control over the migration of the solute in the capillary.

To avoid any issues, the capillary inner walls can be coated with gel polymer that highly reduces these differences in DNA fragment separation.

The polymer used in this study is POP-4™, and the buffer is the TAPS 10x 3-[[1,3-dihydroxy-2-(hydroxymethyl) propan-2-yl] amino] propane-1-sulfonic acid (TAPS).

It contains also pyrrolidinone and urea as denaturant agents. The buffer moreover is stabilized by the addition of ethylenediaminetetraacetic acid 1mM (EDTA) as a chelating agent. The pH of the buffer is 8. from Thermoscientific. It is critical to have a gel polymer and a buffer with a balanced composition and certain pH since these two elements can affect the EOF and therefore the results of the CE.

In the final step of the process, the laser targets the fragments in the detection window. The detection occurs when the laser excites the fluorescent dyes attached to each DNA fragment. Finally, the signals are directed to a detector that can convert them into peaks. The interpretation of the peaks and raw data is made by specific software.

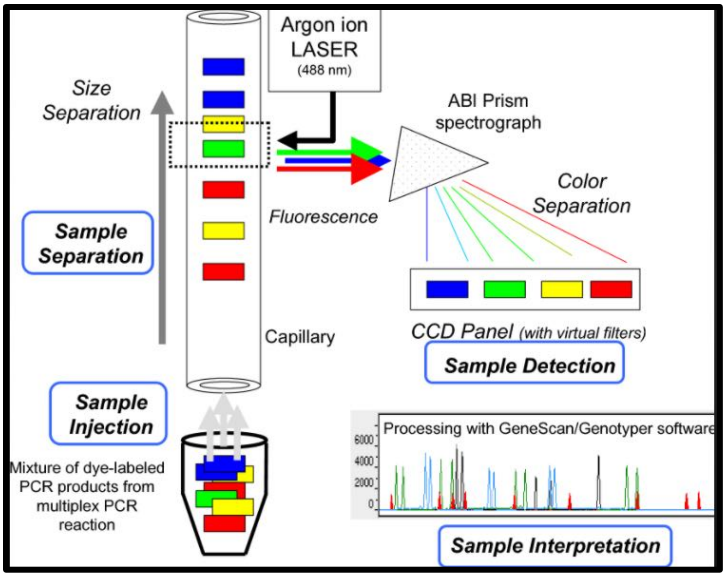


Fig.37 Illustration showing an example of the mechanism of detection using gel electrophoresis. Butler, 2004

Forensic laboratories use several instruments type DNA samples. In this project, the one used was the Genetic analyzer 3130XL produced by Applied Biosystems™ (Foster City, CA).

The protocol for the sample analysis, is automated system is straightforward (Life Technologies technical manual 2012). Briefly, the amplified samples are first electrokinetically injected from their tubes on the sample tray, into a silica glass capillary of a certain length (36 cm in this case) that is filled with a specific polymer able to separate the DNA amplicon fragments according to their size. The voltage applied causes the negatively charged DNA fragments to travel through the capillary to reach the other end, the cathode. One buffer reservoir is present at the anode and at the cathode position, called also inlet and outlet reservoir. During the final step of the migration, the samples encounter the detection window with the laser that can excite the dyes located on one of the two

primers of the STR loci, and the signals obtained are observed in order of their size and represented in the form of electropherograms.

In this research project, the protocol for genotyping was as follows.

1–1.5µl of PCR product 12.5µl Hi-Di formamide and 0.5µl LIZ500 as our internal size standard were combined for each sample. The analysis method used was the HID Fragment Analysis36_POP4_1, the temperature was set at 60°C and the run time at 30 min. The analysis method and run mode used were according to the manufacturer protocol. GeneMapper® ID-X was the software used to create and interpret the DNA profiles.

6.3 Results and Discussion

6.3.1 Saliva

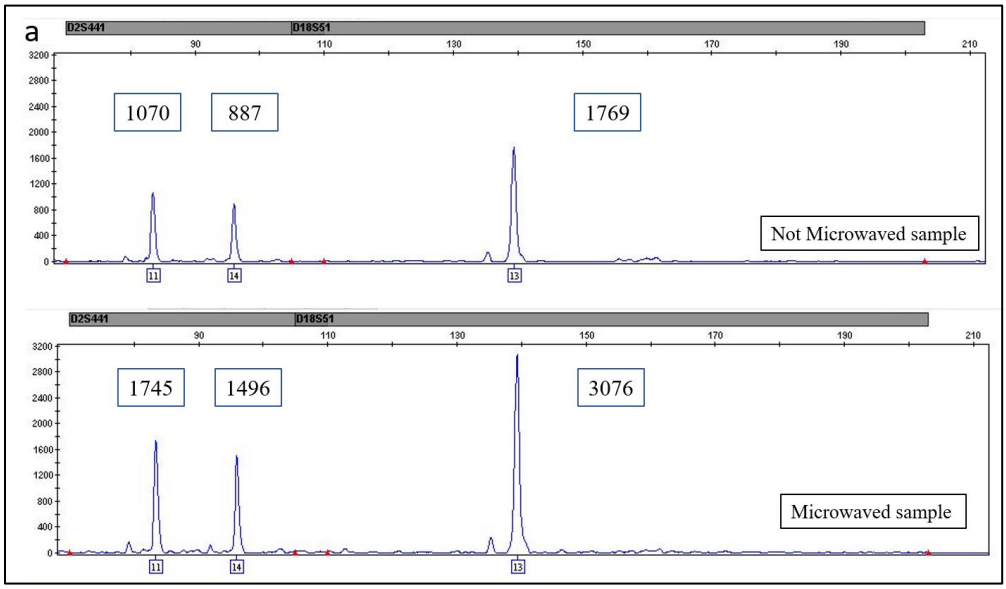
In this section results from genotyping will be explained. As already mentioned in the protocol section, the amplification reaction has been re-designed.

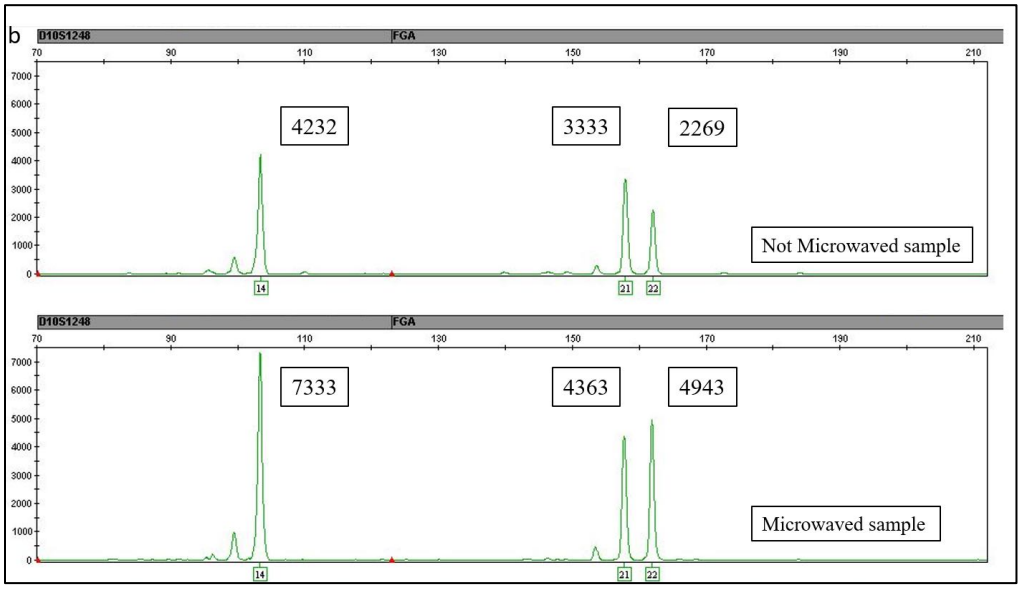
Regarding the saliva samples, several attempts have been made to find the right percentage of dilution that mimics low template samples, resembling the evidence often found at crime scenes. Microwave treatment improved the peak heights of the saliva samples and increased the recovery of those alleles not detected in non- microwaved samples (technically called drop out).

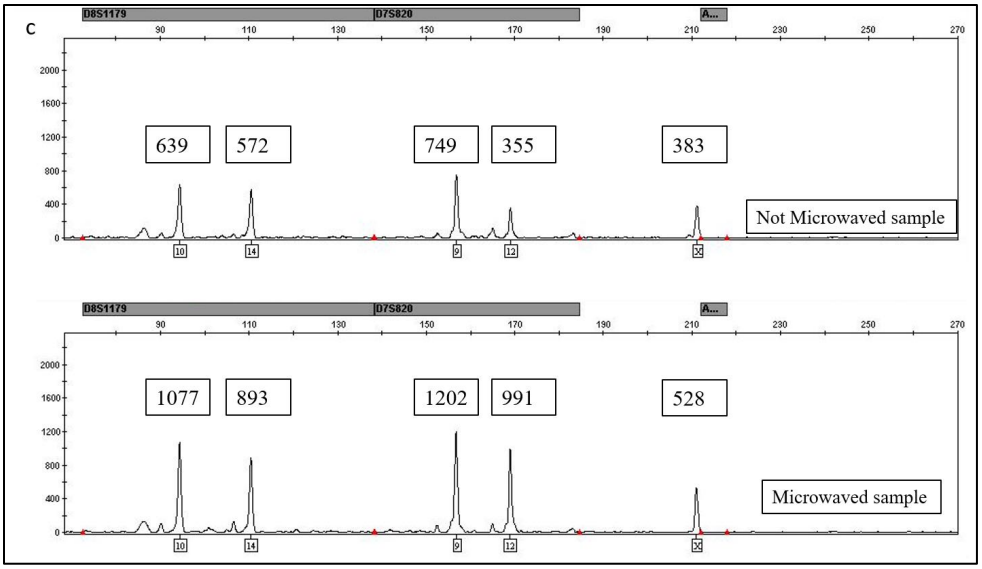
The following electropherograms illustrate a female DNA profile obtained from a 1% saliva sample.

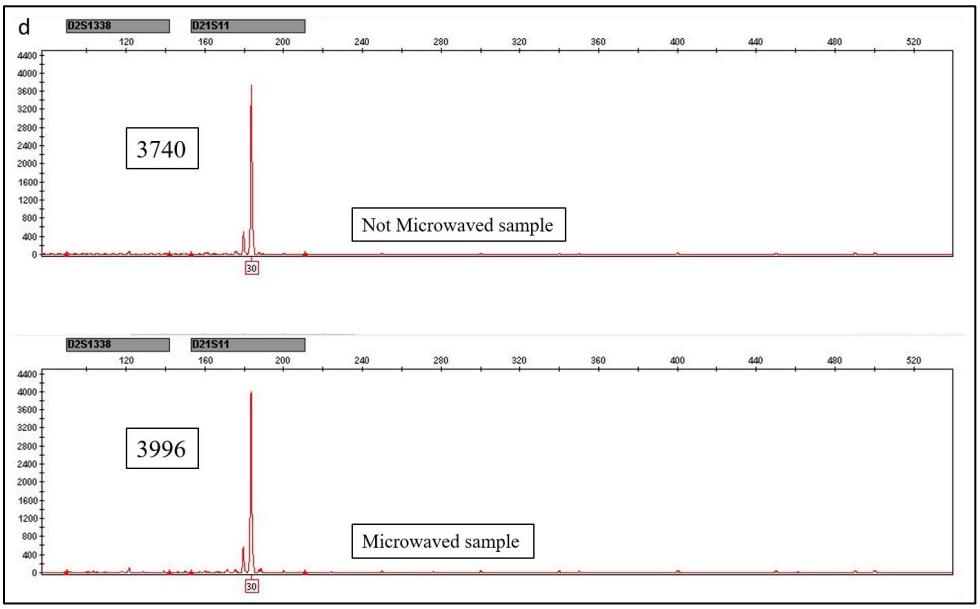
The numbers displayed in the boxes are the peak heights, expressed in Relative Fluorescent Unit (RFU) that are represented on the *y* axis of the electropherogram. The microwaved and not microwaved samples are compared according to each fluorescent dye.

DONOR 1, FEMALE SALIVA SAMPLE DILUTED 1%






c
D8S1179
D7S820
A...
639
572
749
355
383
Not Microwaved sample
1077
893
1202
991
528
Microwaved sample


d
D2S1338
D21S11
3740
Not Microwaved sample
3996
Microwaved sample

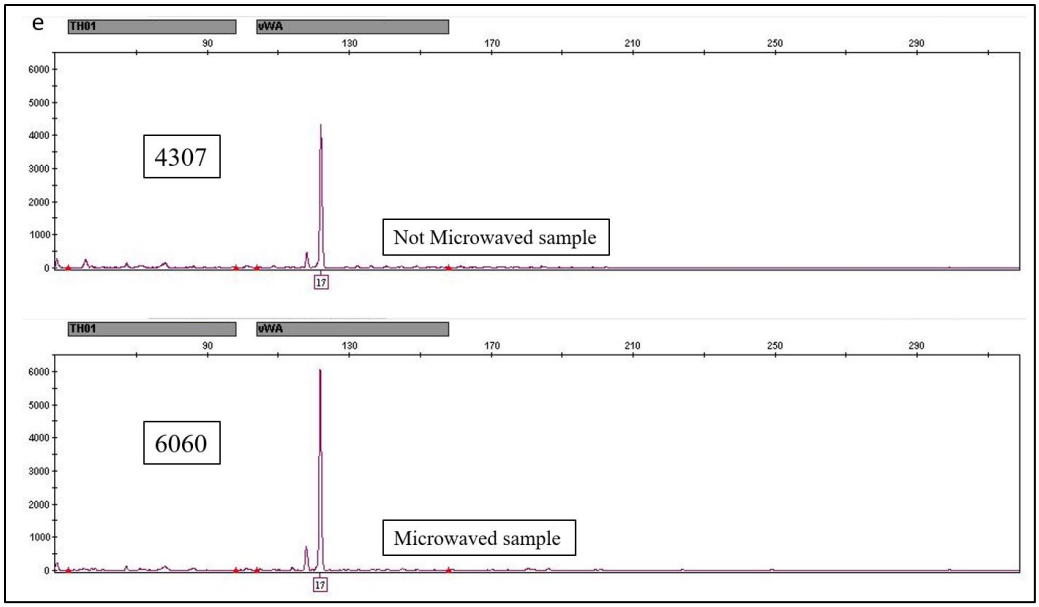
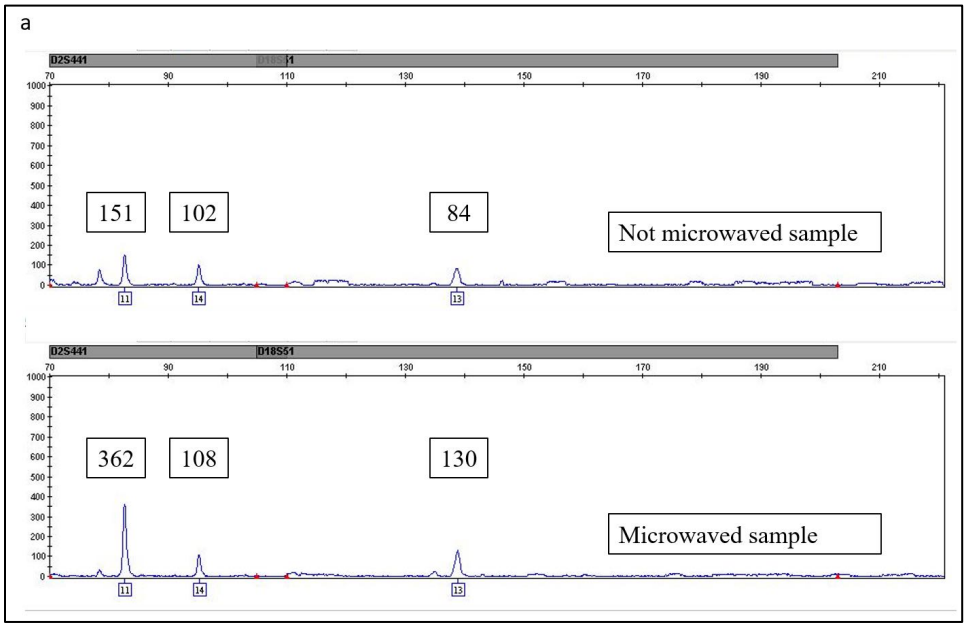
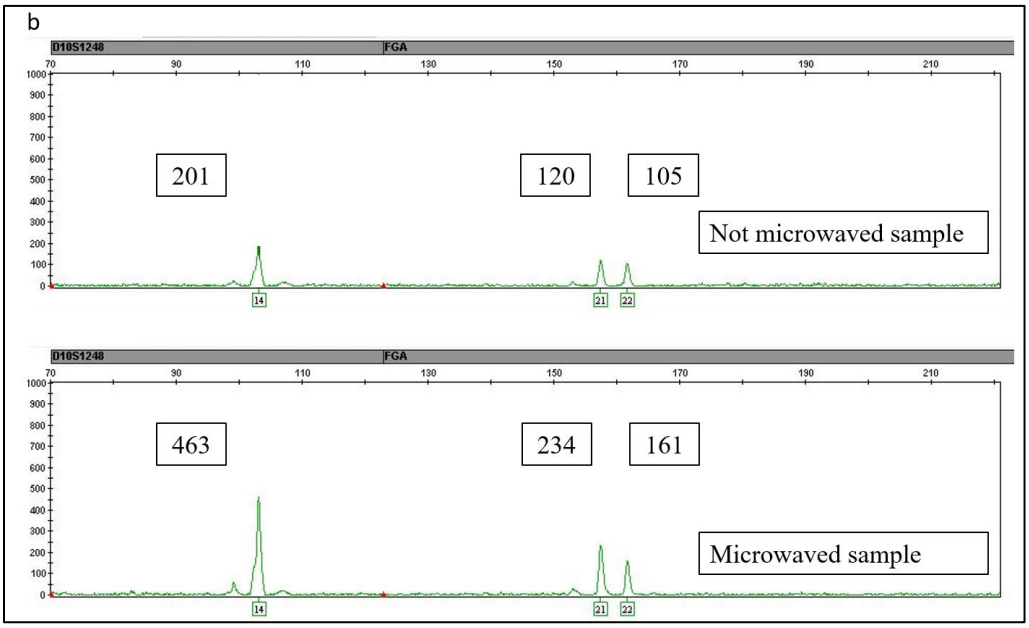


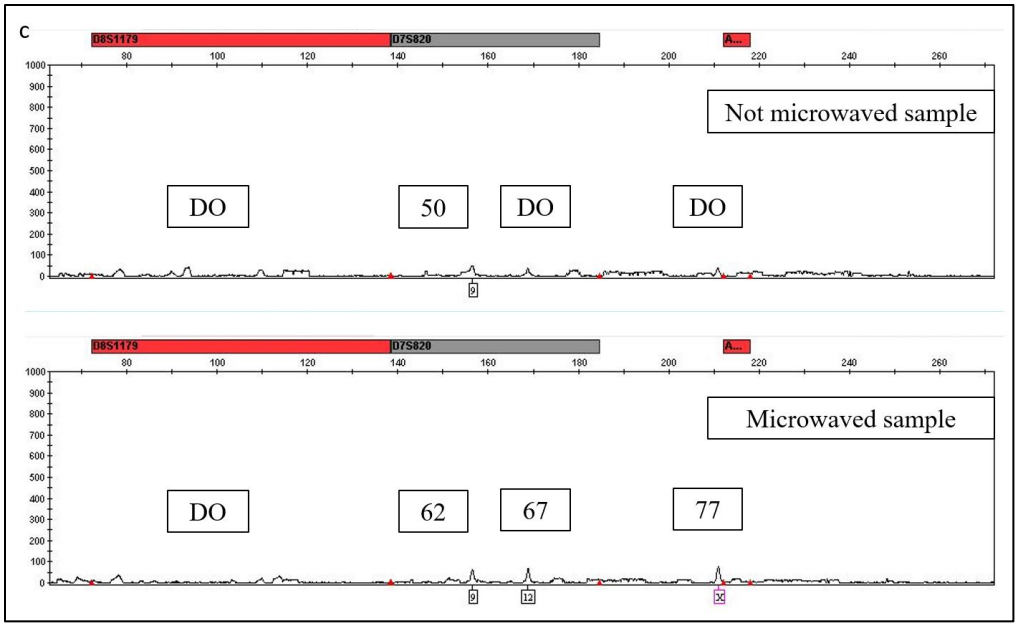
Fig.38 a, b, c, d, and e. Electropherogram of donor 1, female saliva sample diluted 1%. In the boxes, the peak heights in terms of RFU are shown

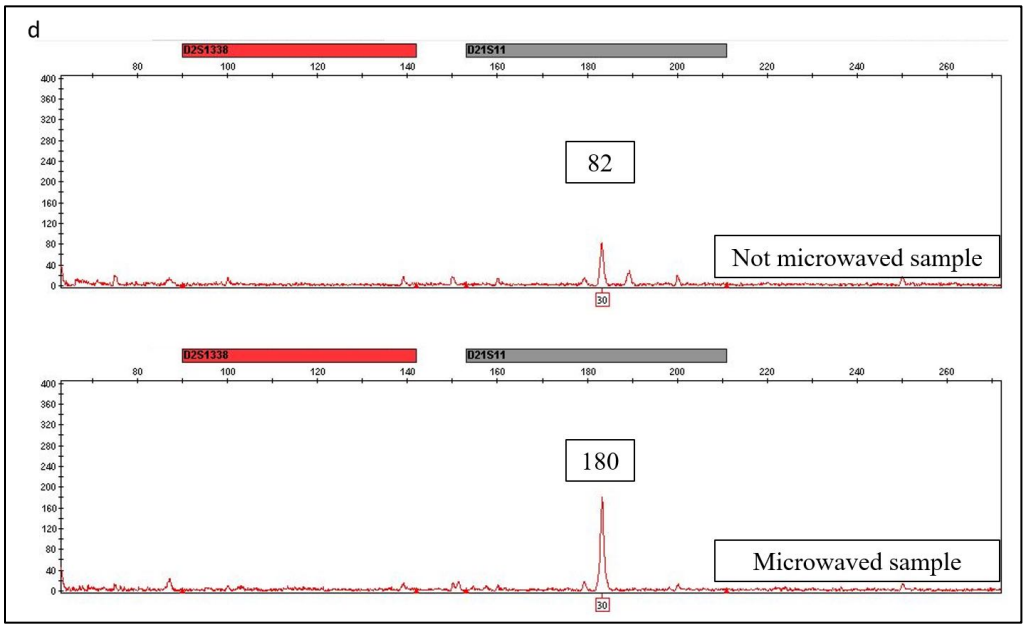
The overall results show higher peaks for all the microwaved loci with an average increase of 68%. Next, (Figures 39, 40 and 41) results obtained from two 1% saliva samples from 2 female donors: one is the same donor (donor 1) from the results shown previously but this represents a fresh sample collected and analyzed on a different day. We state this first to reinforce the concept of the high intra-variability of saliva samples, collected from the same person on different days at the same time we assess the reproducibility of the protocol. The numbers displayed in the boxes are the peak heights, expressed in Relative Fluorescent Unit (RFU) shown on the *y* axis of the electropherogram. The microwaved and not microwaved samples are compared according to each fluorescent dye. The acronym found in some graphic called DO indicates Allele Drop Out, a stochastic effect that may occur when the DNA analyzed is in low amount.

DONOR 1, FEMALE SALIVA SAMPLE DILUTED 1%






c
D8S1179
D7S820
A...
Not microwaved sample
DO
50
DO
DO
Microwaved sample
DO
62
67
77


d
D2S1338
D21S11
82
Not microwaved sample
180
Microwaved sample

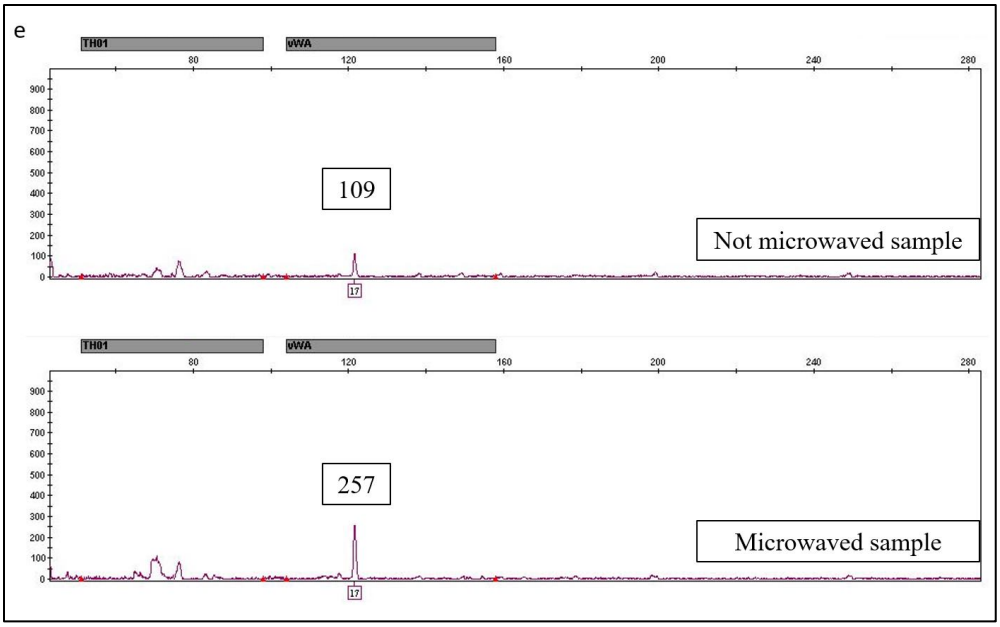
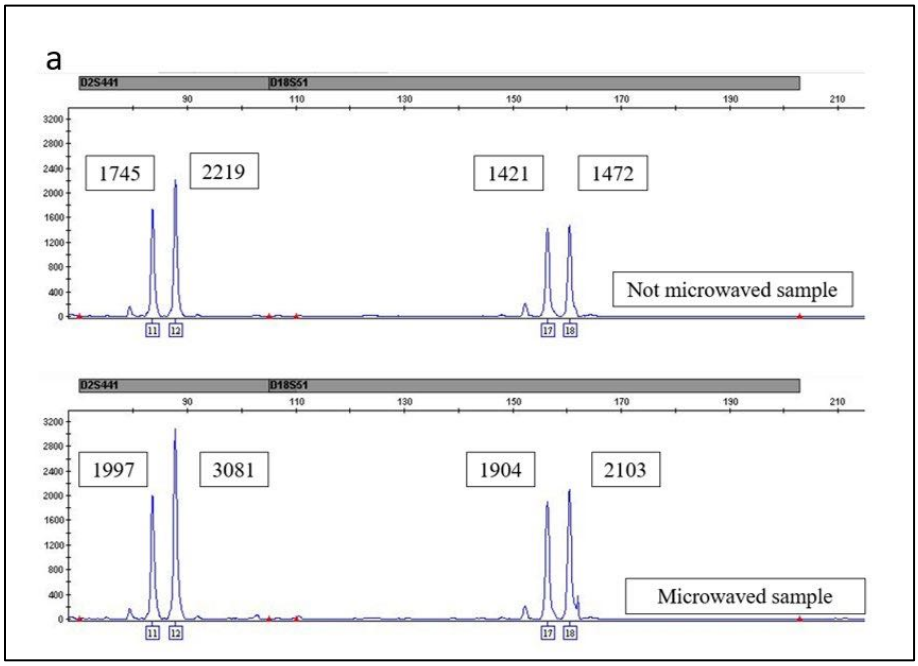
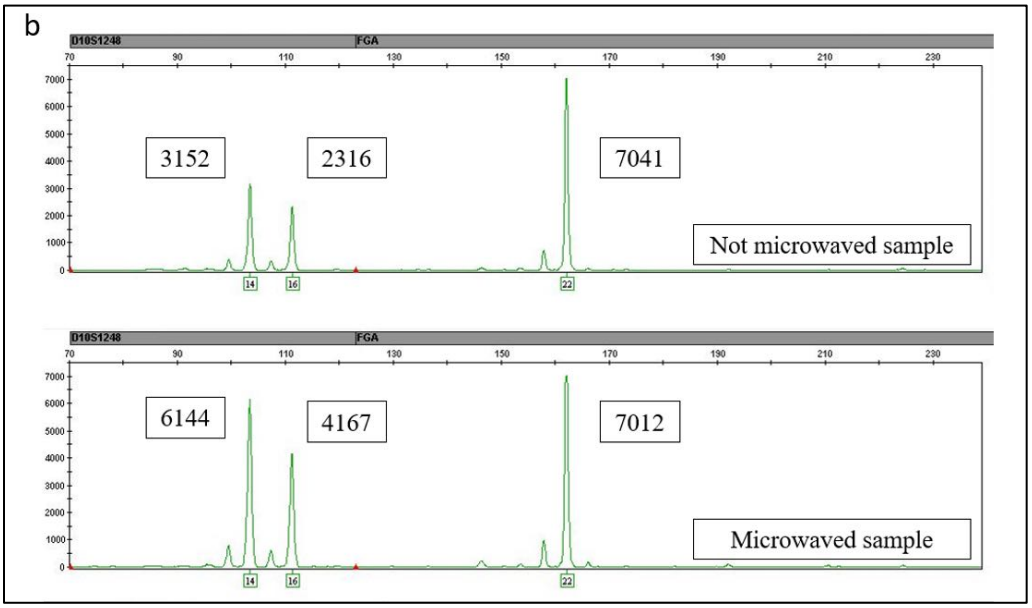


Fig.39 a, b, c, d, and e. Electropherogram of a female saliva sample, donor 1, diluted 1%. In the boxes, the peak heights in terms of RFU are shown

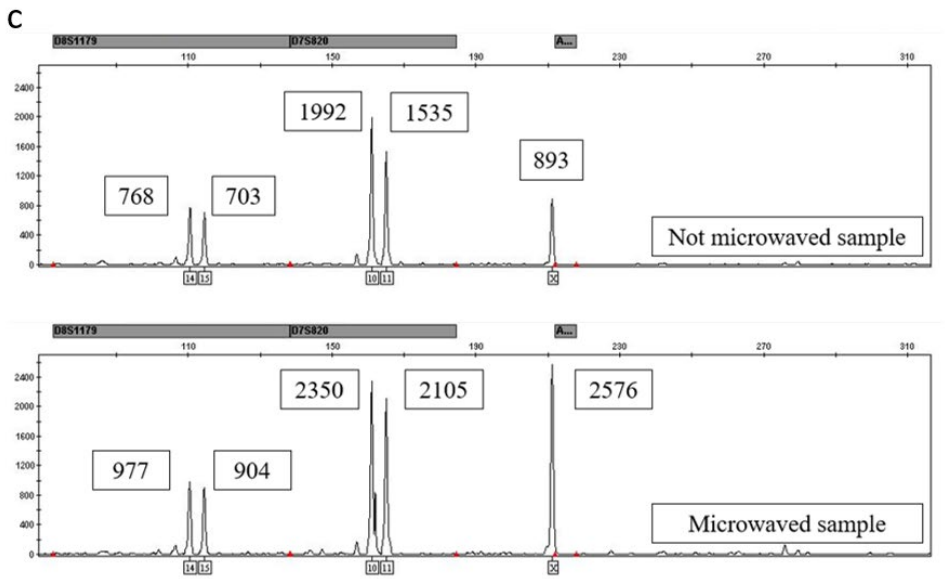
The results demonstrate improved DNA amplicon yields from microwave treated low template samples. While the increase in peak heights between microwaved and not microwaved samples is not always observed for all loci, the average increase in PHs is 84% Following the analysis, testing of the second female donor 1% saliva sample was performed. The analysis was performed on two replicates of not microwaved samples and 2 replicates of microwaved sample

DONOR 2, FEMALE SALIVA SAMPLE DILUTED 1%, REPLICATE 1

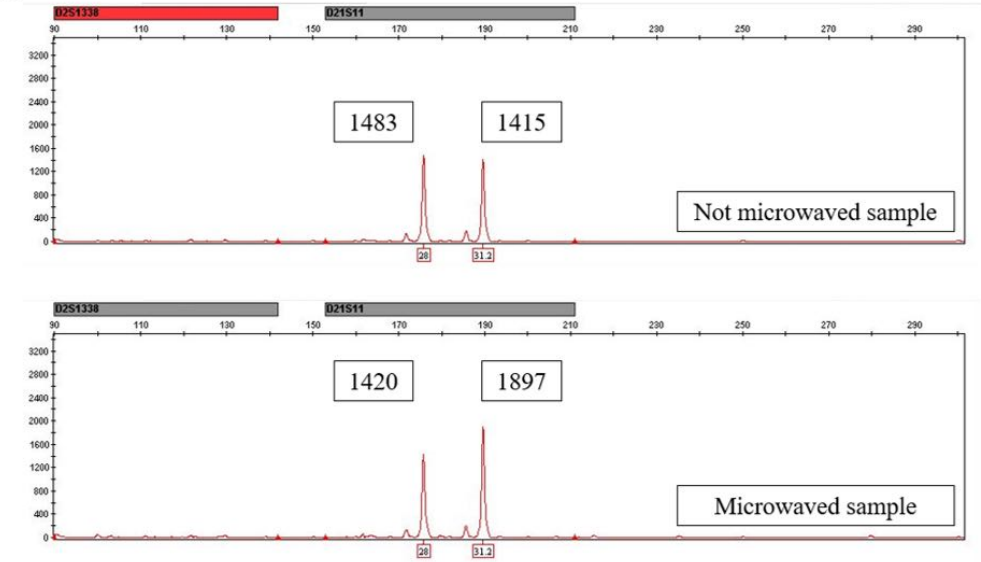




c


D8S1179
D7S820
768
703
1992
1535
893
Not microwaved sample
977
904
2350
2105
2576
Microwaved sample

d


D2S1338
D21S11
1483
1415
Not microwaved sample
1420
1897
Microwaved sample

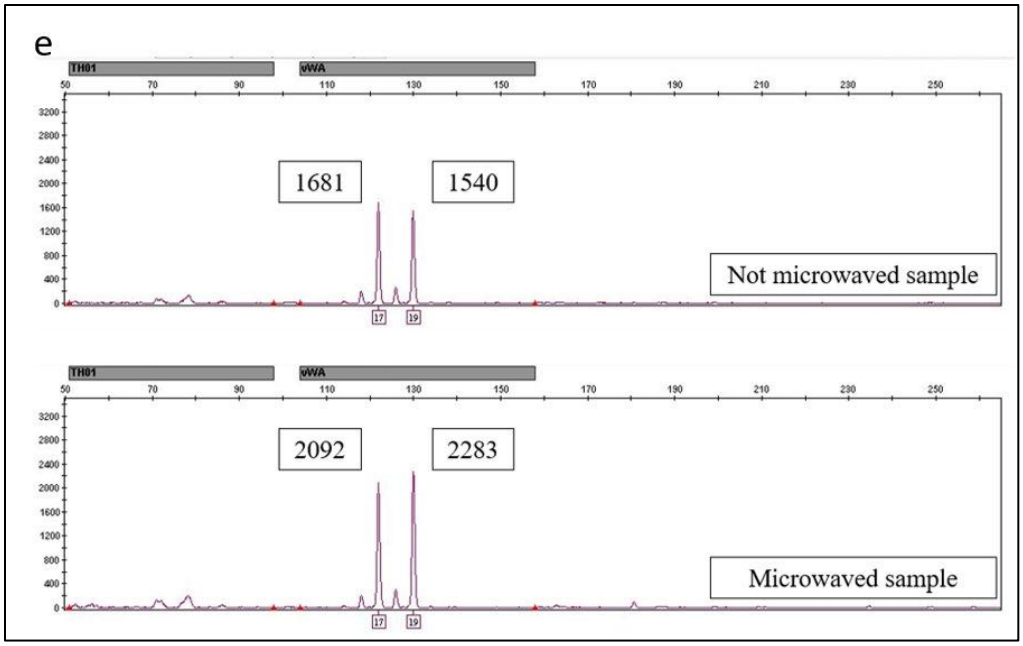
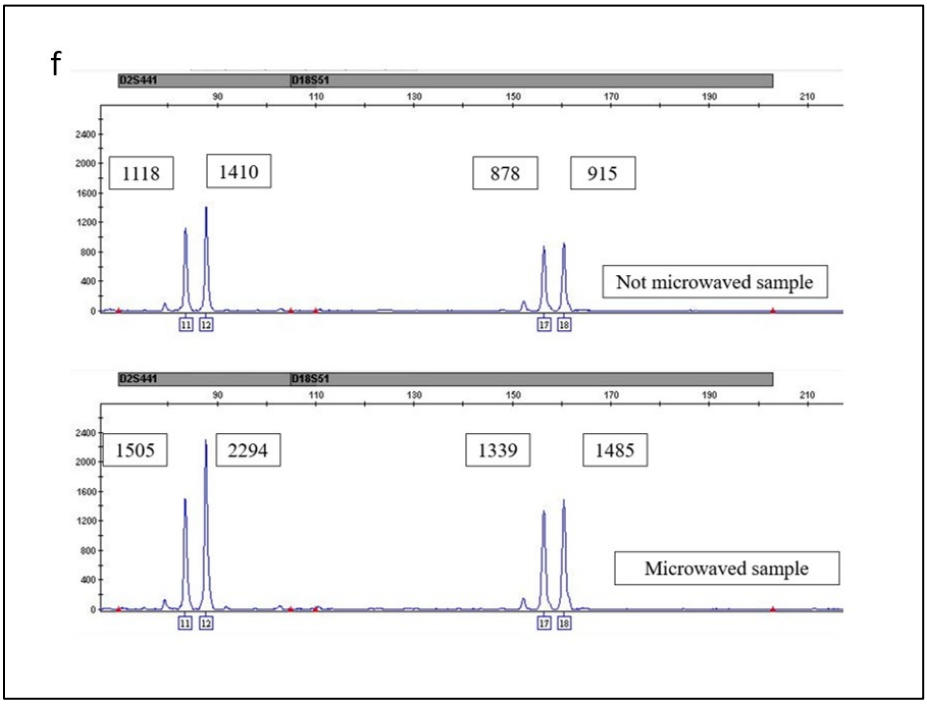
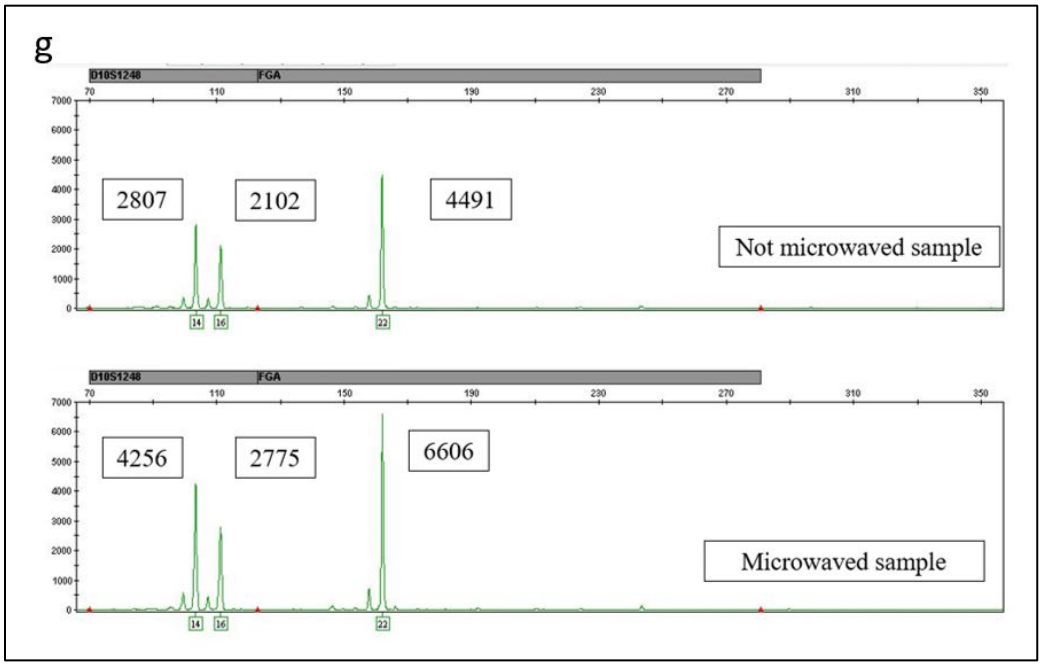


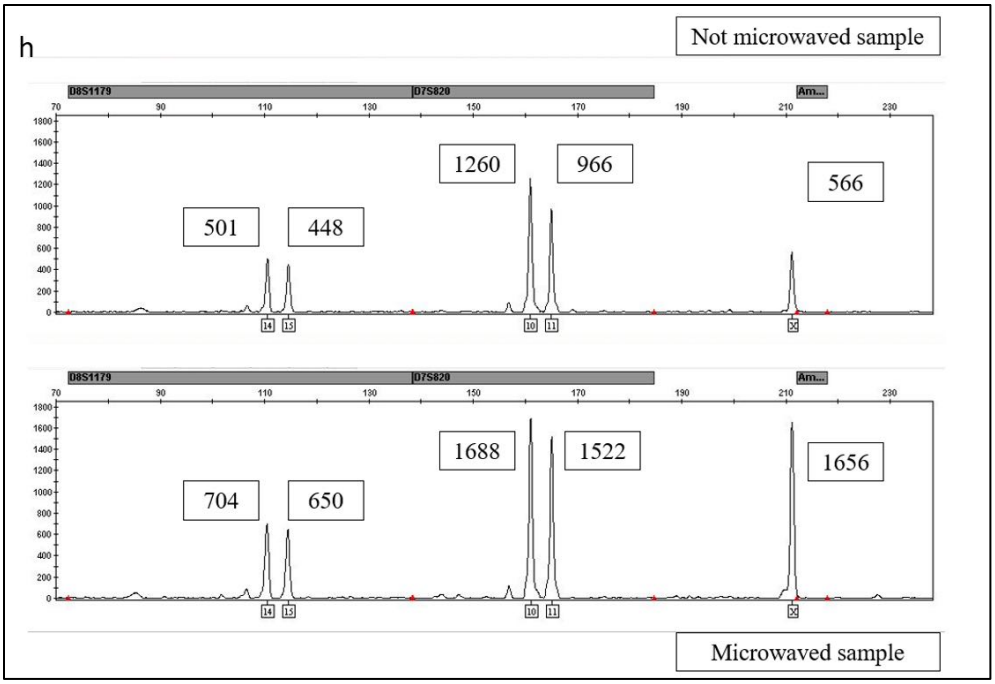
Fig.40 a, b, c, d, and e. Electropherogram of a female saliva sample, donor 2, replicate 1, diluted 1%. In the boxes, the peak heights in terms of RFU are shown

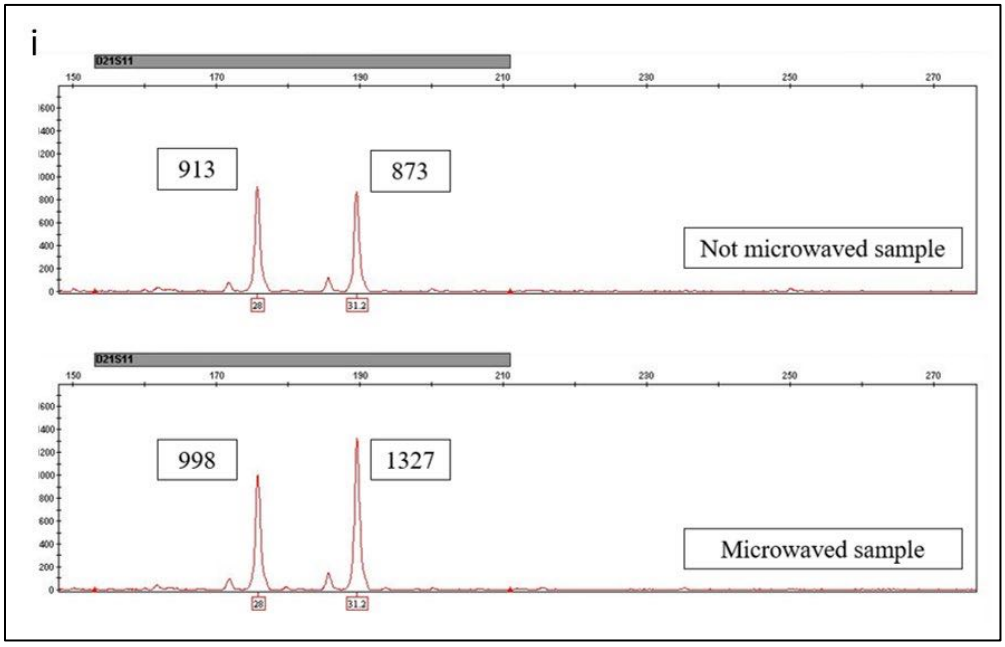
The results from this sample confirmed the increased peak heights and allele detection for microwave treated samples. Except for one locus, D21S11 in which allele 28 is 6 RFU higher in the not microwaved sample than in the microwaved one, all the other alleles increased in peak heights and the overall increase was 44%. Replicate n.2, shown below, confirms the positive trend

DONOR 2, FEMALE SALIVA SAMPLE DILUTED 1%, REPLICATE 2






h
Not microwaved sample
D8S1179
D7S820
Am...
501
448
1260
966
566
704
650
1688
1522
1656
Microwaved sample


i
D21S11
913
873
Not microwaved sample
998
1327
Microwaved sample

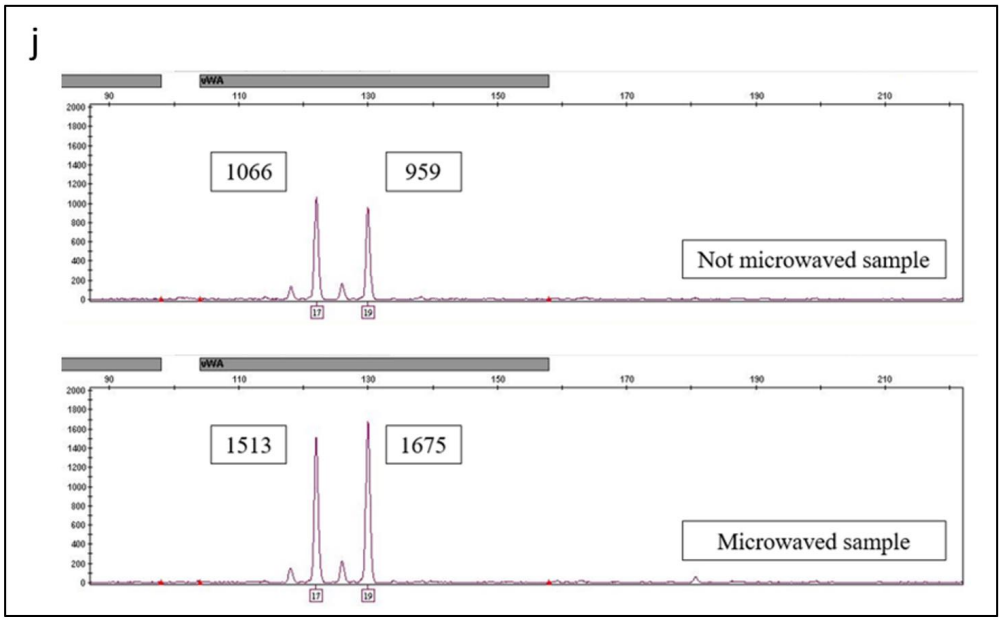


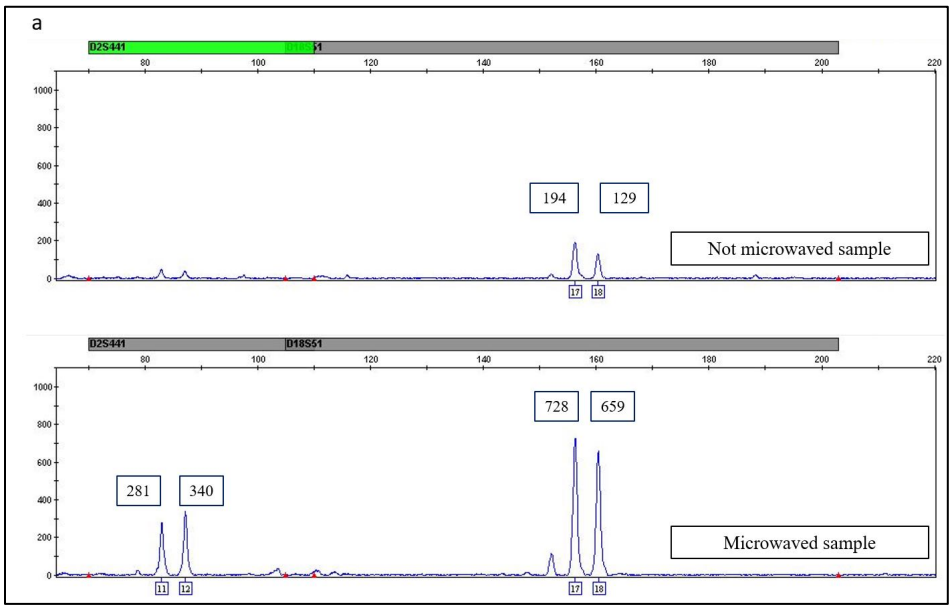
Fig.41 f, g, h, i, and j. Electropherogram of a female saliva sample, donor 2, replicate 2 diluted 1%. In the boxes, the peak heights in terms of RFU are shown

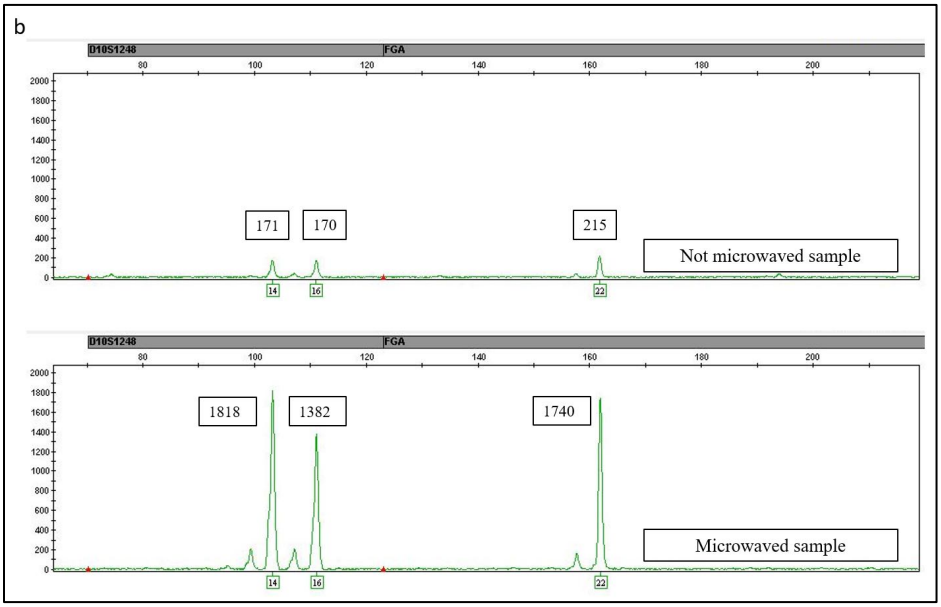
The replicate n.2 shows the same improvement in the microwaved sample as well as replicate n.1. and its overall increase of 56% in peak heights. Therefore, even if internal variability is present between saliva samples, the positive effect of the microwave was shown to be reproducible. Statistical analysis indicates that allele peak height increases and increases in allele detection for the microwave vs non microwave samples is significant. A two-way ANOVA of 1% saliva samples analyzed using the 9 miniSTR multiplex revealed a significant difference in average peak heights between microwaved and non-microwaved samples (F-statistic = 10.53, p-value = 0.011785)

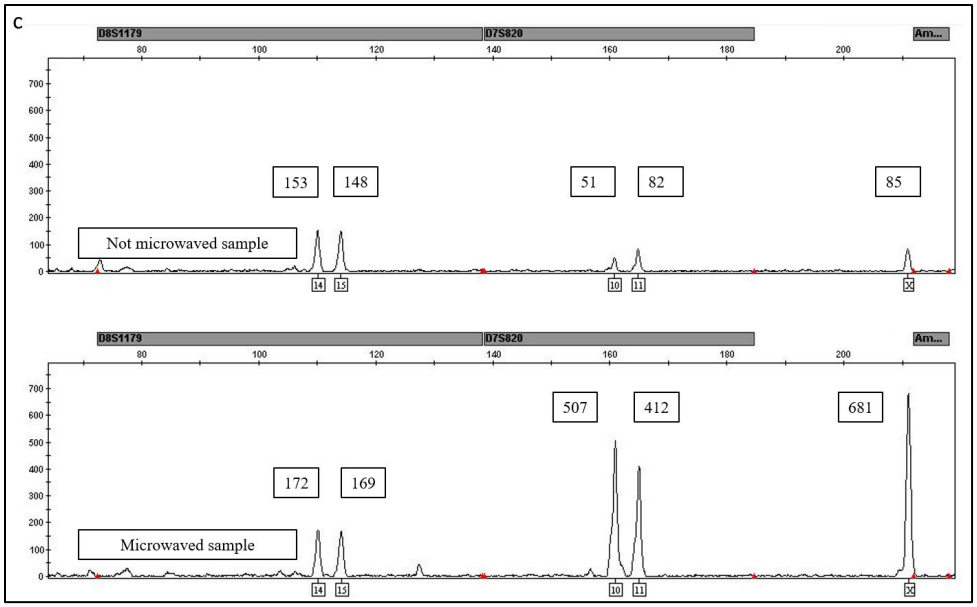
6.3.2 Blood

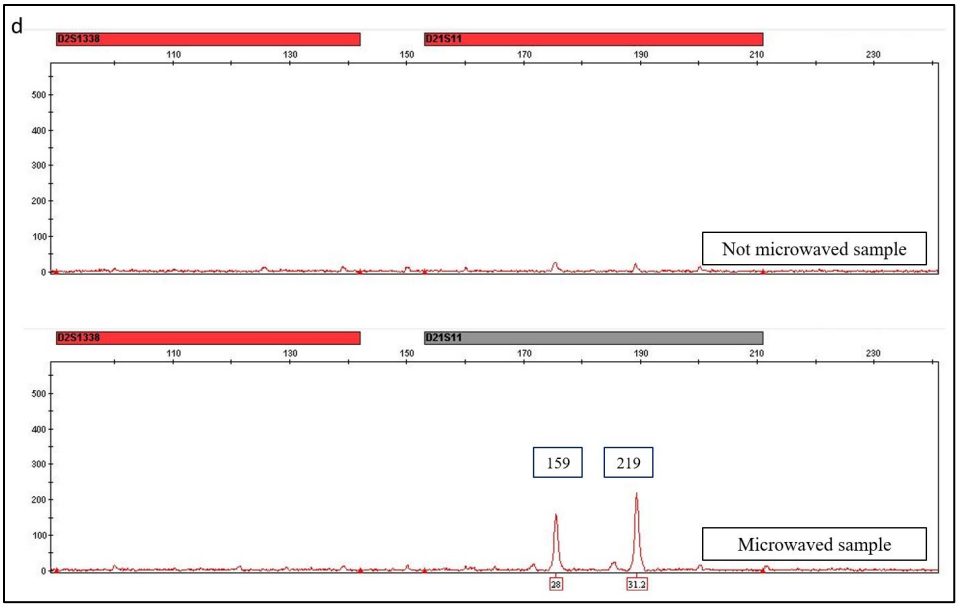
The following electropherograms (Figure 42) show the results obtained from a female 1% blood sample. As for saliva, several attempts were made to determine the best time of microwave exposure. The final time and power setting were set at 40 sec 300 W.

DONOR 2, FEMALE BLOOD SAMPLE DILUTED 1%






c
D8S1179
D7S820
Am...
153
148
51
82
85
Not microwaved sample
507
412
681
172
169
Microwaved sample


d
D2S1338
D21S11
Not microwaved sample
159
219
Microwaved sample

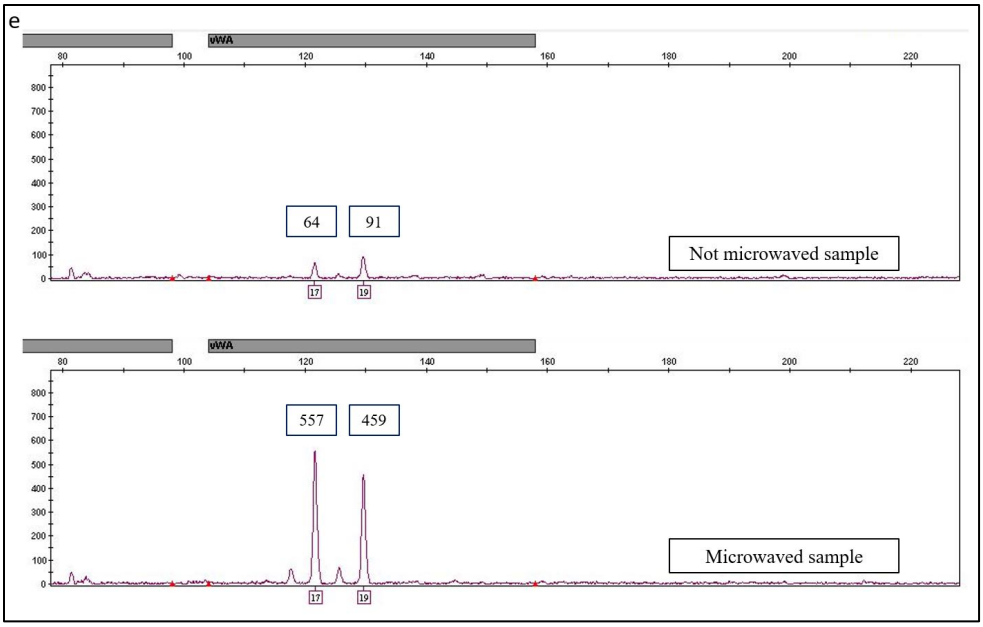


Fig.42 a, b, c, d and e. Electropherogram of donor 2, female blood sample, diluted 1%. In the boxes the peak heights in terms of RFU are shown

STR LOCI	DNA ref	MW PH (ave)	NMW PH (ave)	percentage increase
D2S441	11,12	311	-	**N/A**
D18S51	17,18	694	162	**328%**
D10S1248	14,16	1600	170	**841%**
FGA	22	1740	215	**709%**
AMEL	X,X	681	85	**701%**
D8S1179	14,15	171	151	**13%**
D7S820	10,11	460	66	**597%**
D21S11	28,31.2	189	-	**N/A**
vWA	17,19	508	77	**560%**

Table 7. Results obtained from the blood sample diluted 1%, peak heights average, and percent of increase are shown

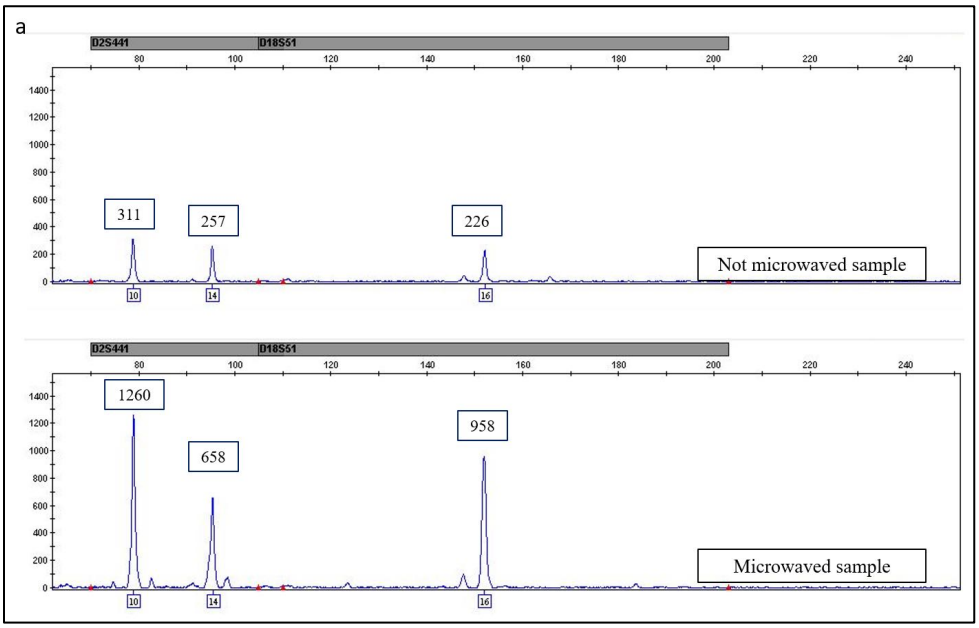
Microwaved vs non microwaved blood sample results demonstrated increased peak heights on average of 522% (range 12-963 RFU) (Table 7). Note that the increase in amplification

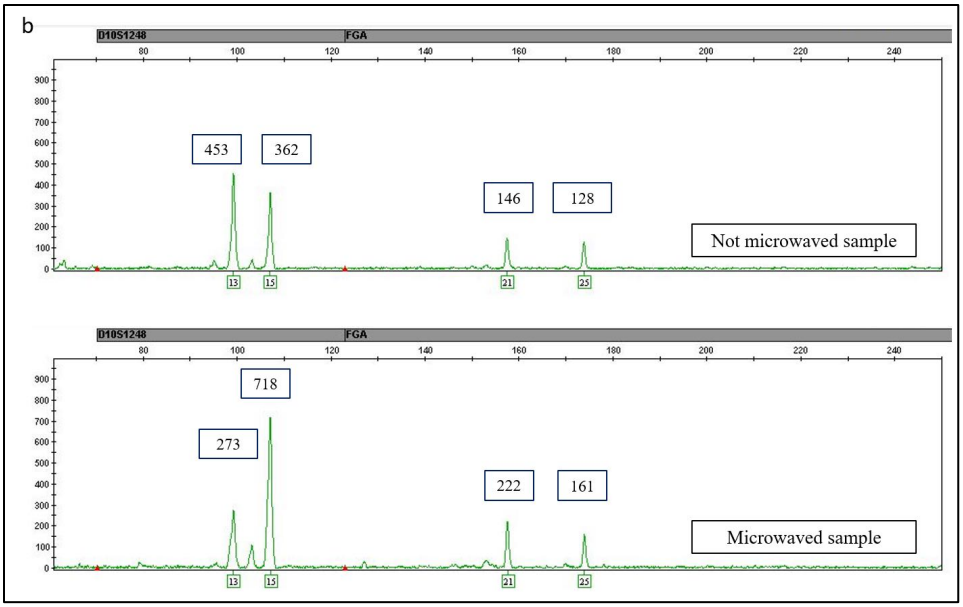
for the blood sample shown represents the highest increase observed A two-way ANOVA of 1% blood samples analyzed, revealed a significant difference in average peak heights based on microwave status (F-statistic = 7.997, p-value = 0.0089).

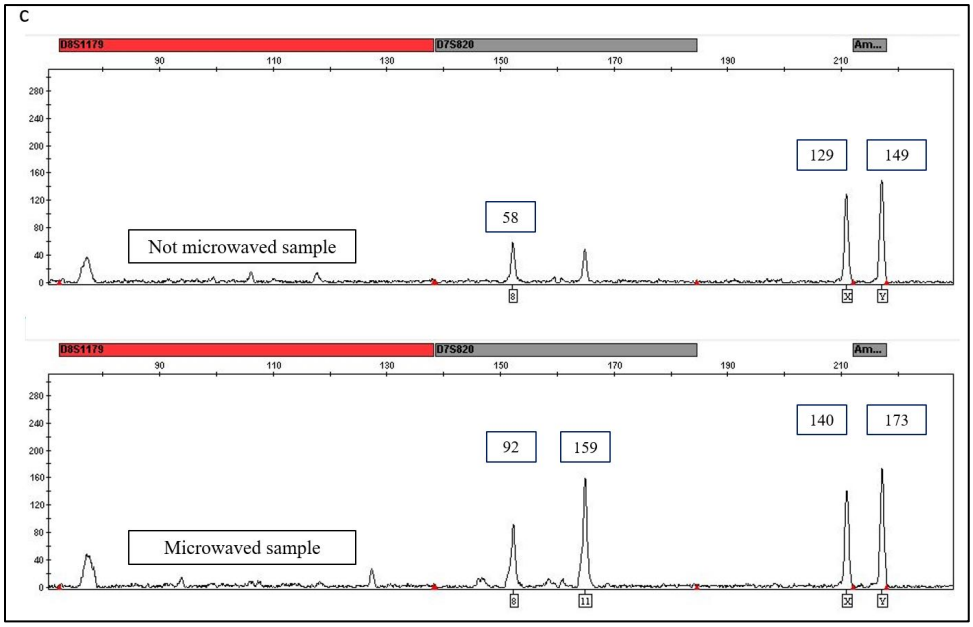
6.3.3 Semen

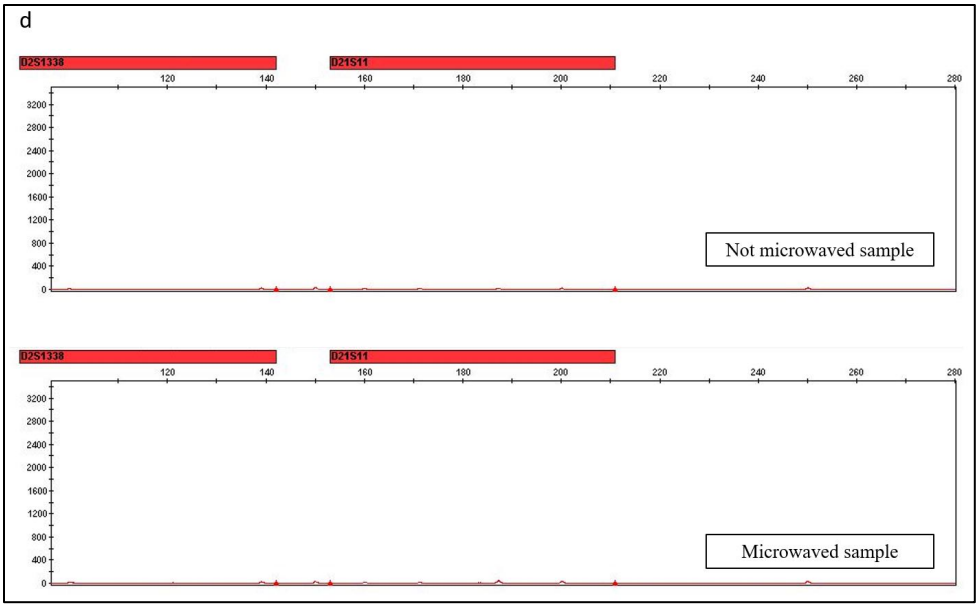
Experiments examining semen samples using microwave treatment (40 seconds 300W) vs non-microwave using 0.5% dilution of semen (10ul) demonstrated increased peak heights on an average of 100% as well as an increase in allele detection. Results for semen samples produced very high increases as observed in the other body fluids too. No significant difference in the peak heights for not microwaved versus microwaved 0.5% semen were observed, possibly due to increased dropout.

DONOR 3, MALE SEMEN SAMPLE DILUTED 0.5%




b
D10S1248
FGA
453
362
146
128
Not microwaved sample
D10S1248
FGA
718
273
222
161
Microwaved sample


c
D8S1179
D7S820
Am...
129
149
58
Not microwaved sample
D8S1179
D7S820
Am...
140
173
92
159
Microwaved sample


d
D2S1338
D21S11
Not microwaved sample
D2S1338
D21S11
Microwaved sample

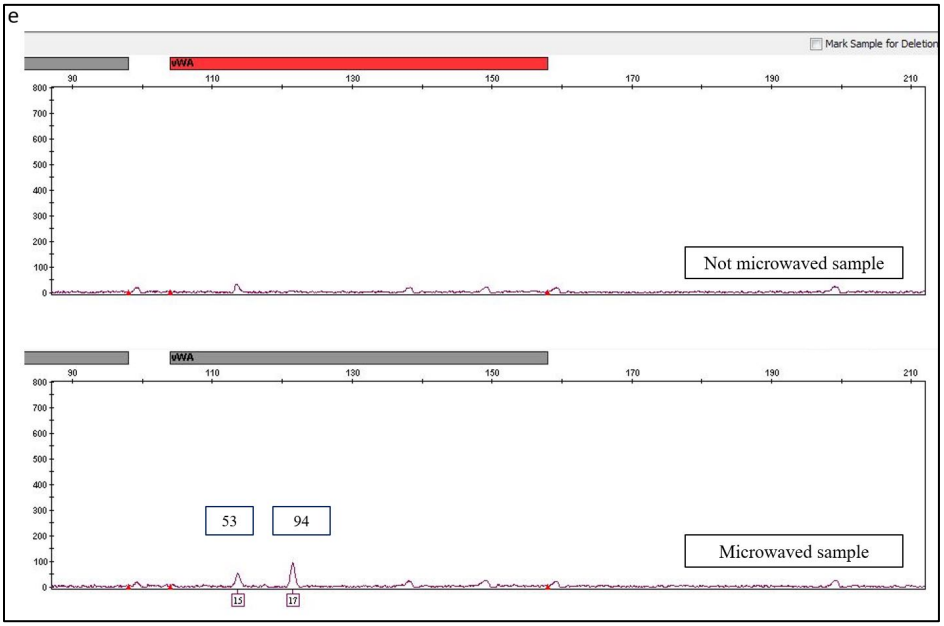

e
Mark Sample for Deletion
vWA
Not microwaved sample
vWA
53
94
Microwaved sample

Fig.43 a, b, c, d, and e. Electropherogram of the male semen sample, donor 3, diluted 0.5%. In the boxes the peak heights in terms of RFU are show

STR loci	DNA ref	MW PH (RFU) ave	NMW PH (RFU) ave	percentage increase
D2S441	10,14	959	284	**238%**
D18S51	16	958	226	**323%**
D10S1248	13,15	496	408	**22%**
FGA	21,25	192	137	**40%**
AMEL	X, Y	156	139	**12%**
D8S1179	10,13	-	-	**N/A**
D7S820	8,11	126	58	**117%**
D21S11	27,31	-	-	**N/A**
vWA	15,17	74	-	**N/A**

Table 8. Results obtained from the semen sample diluted 0.5%, peak heights average, and percent of increase are shown

6.3.4 Mixtures

The effect of microwave treatment on a mixture sample made with saliva and semen, at several ratios were attempted. In Figure 38 the results for saliva/semen mixture ratio 1:9 are shown.

In this paper, the analysis between a female saliva sample and a semen sample is shown.

In the table, the genotypes of the two reference DNA used for this study are listed.

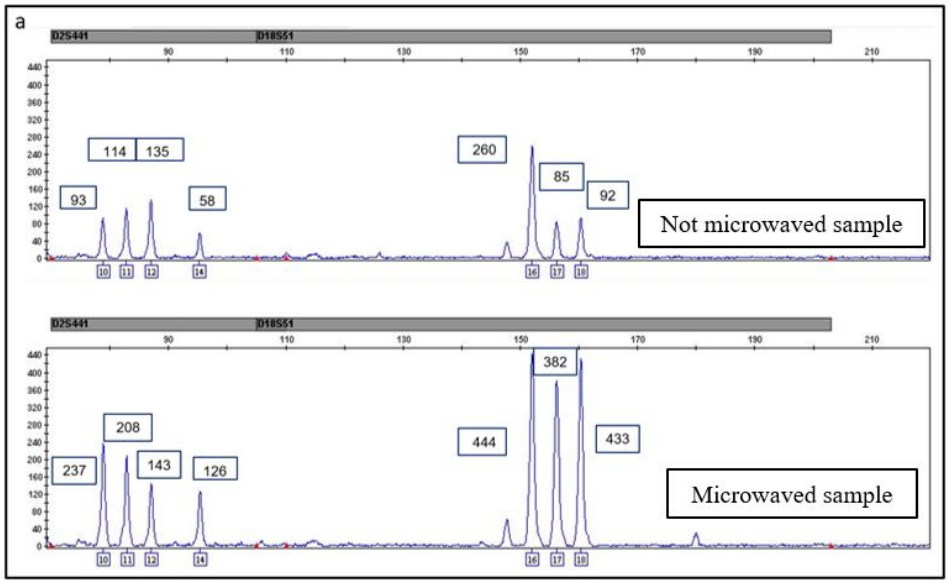
Microwave treatment resulted in recovery of alleles in those loci that had dropped out in the not microwaved sample. In Figure xxx are the results from the same DNA samples in a mixture 1:1 ratio are shown.

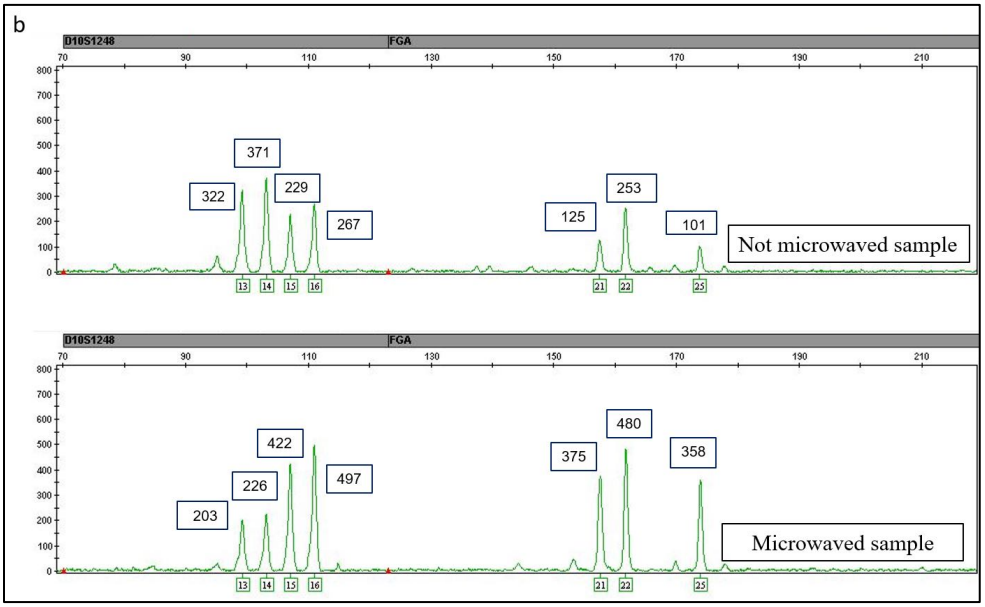
Although the results for the 1:1 mixture ratio do not show a balance between the male and female fraction, the treatment with microwave allowed the recovery of 7 alleles not present in the not microwaved sample.

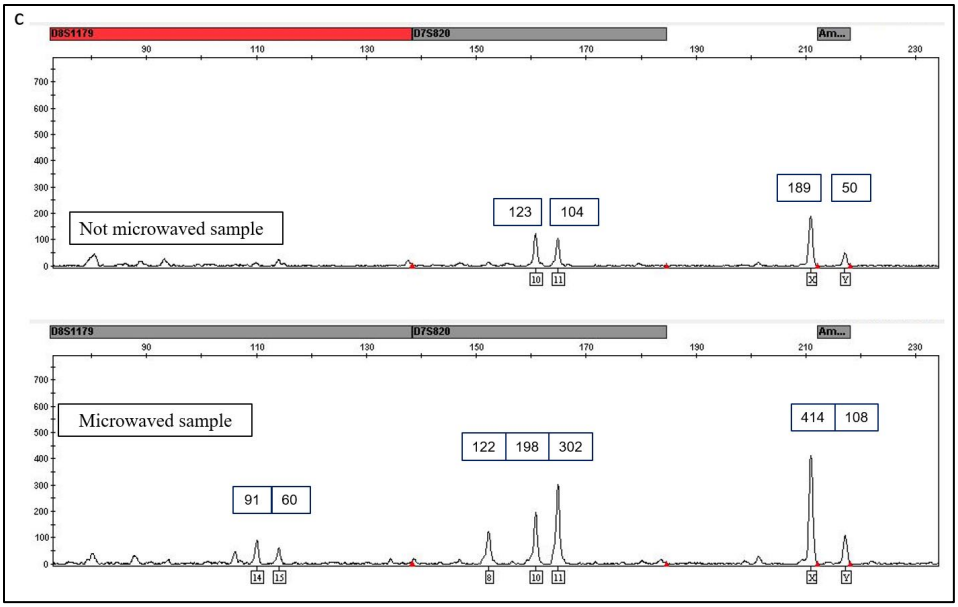
STR	FEMALE DNA	MALE DNA
D2S441	11,12	10,14
D18S51	17,18	16
D10S1248	14,16	13,15
FGA	22	21,25
AMEL	X, X	X, Y
D8S1179	14,15	10,13
D7S820	10,11	8,11
D21S11	28,31.2	27,31
VWA	17,19	15,17

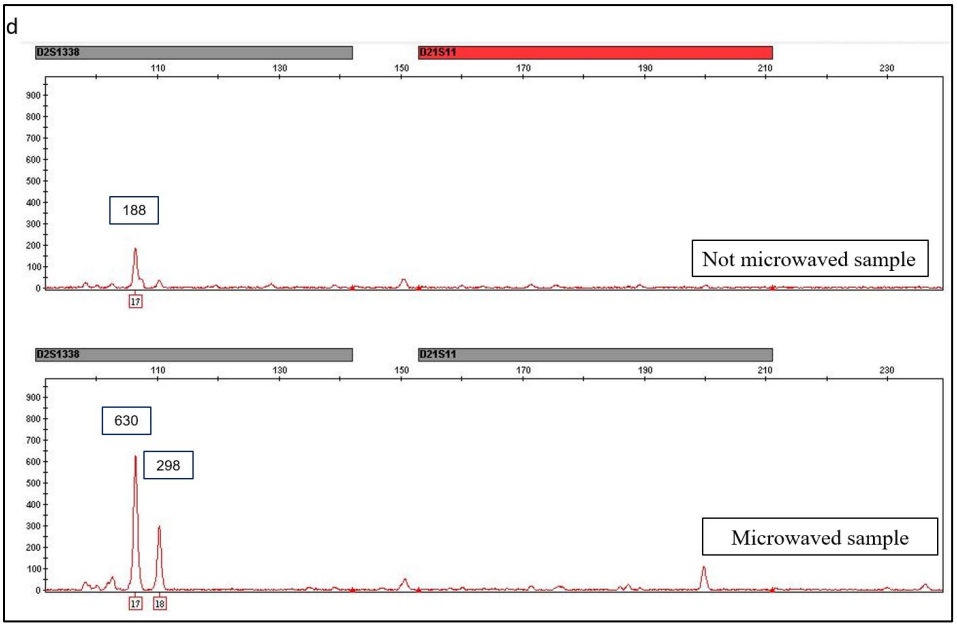
Table 9. Genotype of the mixture contributors; female saliva and male semen

DONOR 2 AND 3, MIXTURE SAMPLE RATIO FEMALE SALIVA- MALE SEMEN 1:9






c
D8S1179
D7S820
Am...
Not microwaved sample
123
104
189
50
Microwaved sample
91
60
122
198
302
414
108


d
D2S1338
D21S11
188
Not microwaved sample
630
298
Microwaved sample

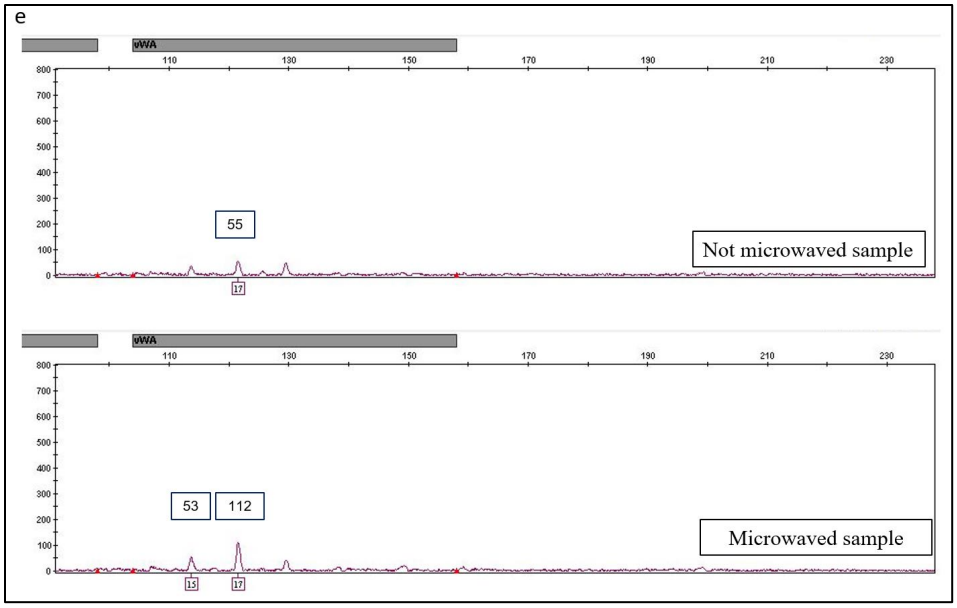
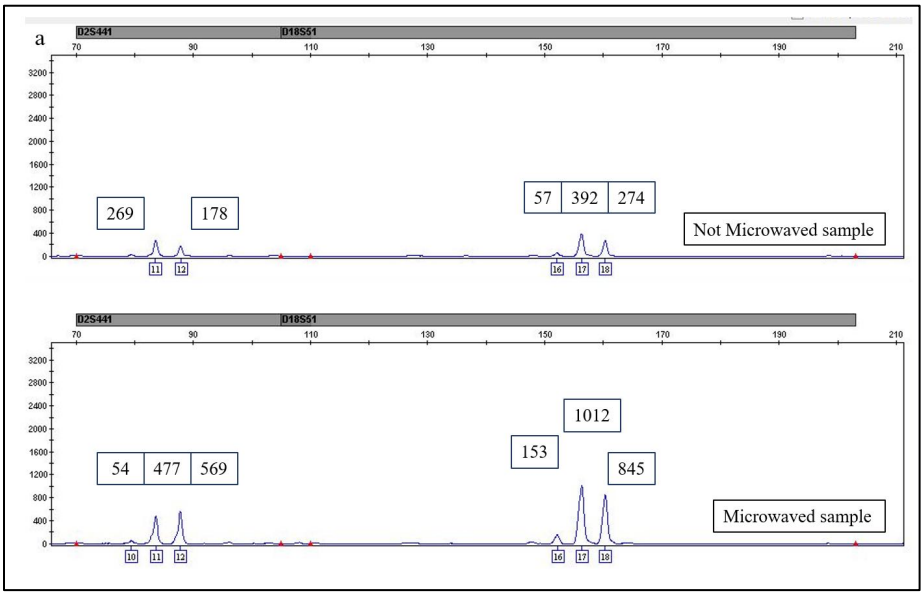
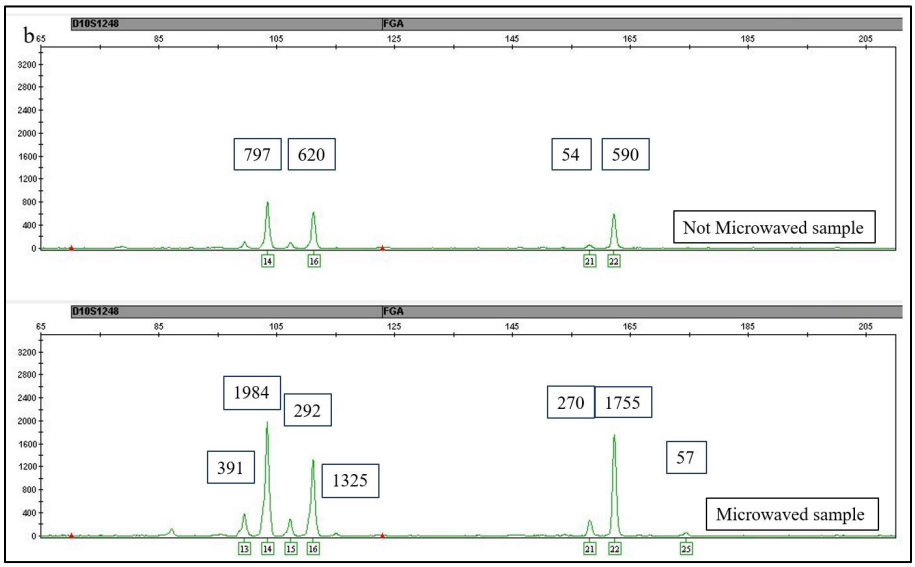
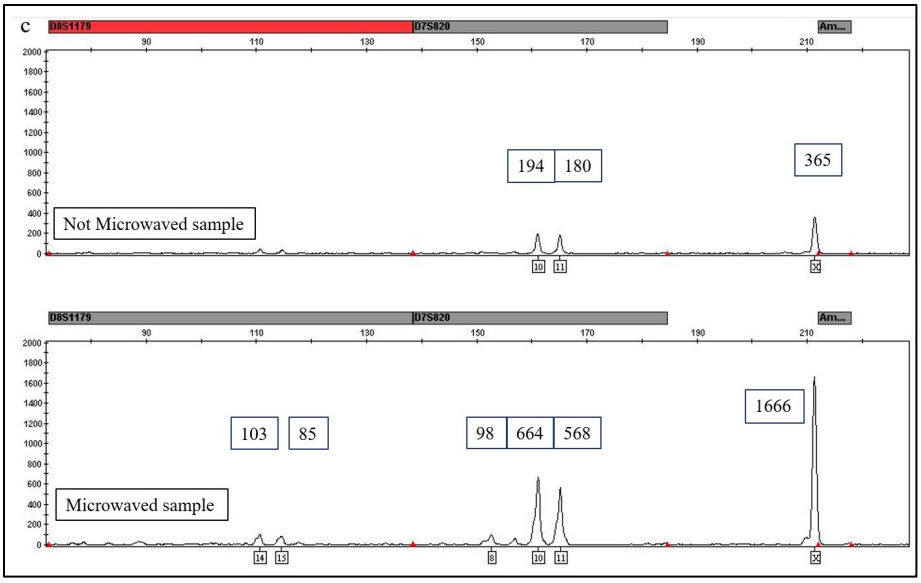


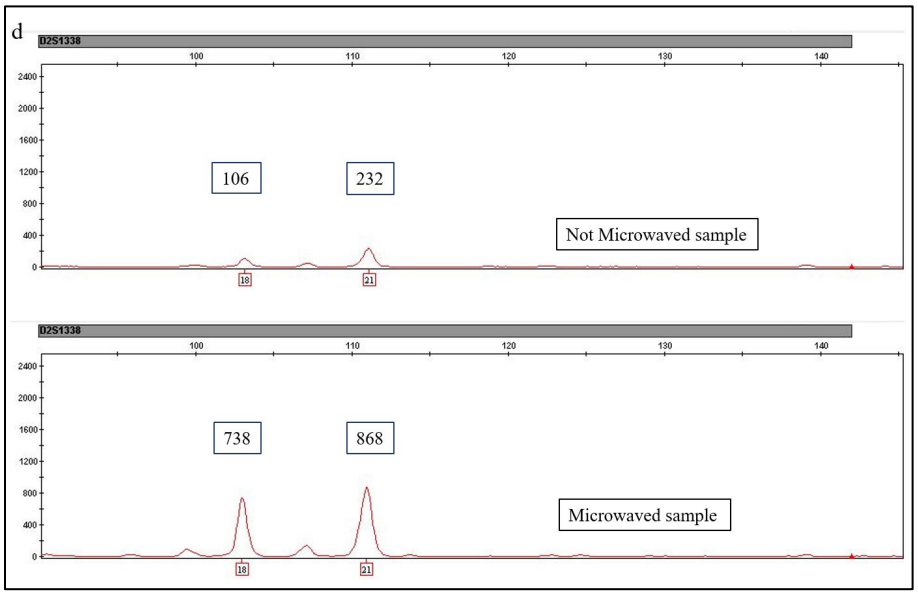
Fig.44 a, b, c, d, and e. Electropherogram of mixture 9:1, female saliva, and male semen. In the boxes the peak heights in terms of RFU are show

DONOR 2 AND 3, MIXTURE SAMPLE RATIO 1:1






c
D8S1179
D7S820
Am...
194
180
365
Not Microwaved sample
103
85
98
664
568
1666
Microwaved sample


d
D2S1338
106
232
Not Microwaved sample
738
868
Microwaved sample

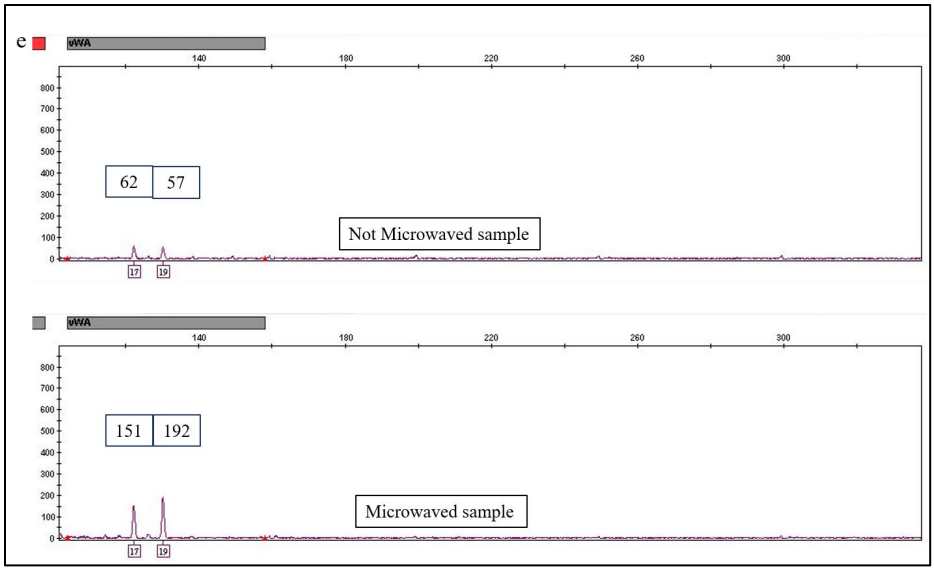


Fig.45 a, b, c, d, and e. Electropherogram of mixture 1:1, female saliva, and male semen. In the boxes the peak heights in terms of RFU are shown

Overall, all the results we obtained from samples genotyping, applying the microwave extraction and the PCR protocol developed, demonstrated the efficacy of the microwave treatment for all the body fluids analyzed. The positive effect of the microwave extraction is not only related to the increase of allele peak height but also to the recovery of those alleles that did not amplify in the not microwaved samples.

CHAPTER 7: Collaborative research with NFSTC, MDPD, and PBSO

In parallel with our analysis at FIU, three external laboratories tested saliva samples exposed to our microwave protocol. These laboratories analyzed the samples on their own RapidHIT instrumentation to corroborate our results.

NFSTC used its microwave to calibrate and perform the analysis. For the Palm Beach Sheriff Office (PBSO) and Miami Dade Police Department (MDPD), we microwaved the swabs and sent swabs via overnight mail. This section describes the results from the three laboratories. At NFSTC, after the calibration of their own microwave oven, several tests were performed. First replicates of saliva samples diluted 1% microwaved and not microwaved were analyzed, and the results showed that the percentage of alleles recovered for the microwaved samples was 92% compared to the 78% of the not microwaved ones. In addition, mock touch samples on a gun were created, collected on a cotton swab and microwaved. The results indicated an increase in recovery of alleles microwaved compared to the sample not microwaved. Testing of dry versus wet swab processing was also performed. The dried samples demonstrated an increase in allele peaks compared to the wet ones. Finally tests on already processed swabs were performed. An increase in recovery of the alleles was observed indicating DNA remains in the already processed swabs indicating rerunning these processed swabs may be useful to up to 4 times resulting in a more complete composite profile.

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MDPD analyzed two replicates of saliva 0.05% diluted and microwaved at FIU according to the protocol developed. Microwaved swabs resulted in an average recovery of 9 and 12 alleles vs not microwaved 4 and 3. MDPD reprocessed microwaved swabs resulted in an average recovery of alleles 5 and 6 and not microwaved alleles 8 and 1.

PBSO analyzed two replicates of saliva 0.05% diluted and microwaved at FIU. Test results on replicate samples demonstrate the increased recovery of saliva samples for the microwaved samples vs not microwaved. Some of these results are summarized in Table 10.

	Not microwaved	**Microwaved**
PBSO	4	**8**
	6	**12**
MDPD	4	**9**
	3	**12**

Table 10 Summary of MDPD and PBSO laboratory results

Overall, all the three external laboratories obtained the same results on all the microwaved and not microwaved samples, corroborating our results and support our conclusion on the applicability of the microwave oven to increase the yield of DNA in forensic laboratory testing.

CHAPTER 8: Conclusion and Future work

In this research project, a novel extraction protocol for forensic samples using a microwave oven was developed. Methods were developed utilizing a commercial microwave oven for sample pretreatment and validated using a series of experiments with saliva samples. These tests involved experimental tests of extraction efficiency using different buffer compositions. Tests involved quantitative analysis, temperature measurements and tests of microwave intensity and sample location. A homemade PCR multiplex of 9miniSTR was developed and optimized for all the body fluids analyzed.

The results obtained indicate that the microwave treatment can provide a fast, easy, non-toxic, and inexpensive method for sample extraction compared to the common methodologies in use. Microwave exposure may also decrease the effect of inhibition, initiates cell lysis and increases DNA yield, particularly for low template samples, resulting in more complete profiles after the treatment. This method should provide a useful tool in forensic genomic testing when very low-level, and degraded samples are encountered. Furthermore, the use of microwave treatment combined with direct PCR impacts a wide variety of research in medical and environmental analysis in which rapid and fieldable sample extraction is necessary.

More in-depth investigations of some other interesting aspects about the use of microwave treatment and its direct effect on the cell and in the quantitative analysis should be done. Previous work has clearly demonstrated the effect of the microwave treatment on bacteria or spore extraction using microscopy and subsequent genetic analysis. In this project we have demonstrated the direct effect of the microwave exposure on the lysis of saliva cells.

Future research could examine this process over a wider variety of DNA extractions. Moreover, there is very little in the published literature on the direct amplification of saliva samples due to variability in recovered cells in saliva from the same individual. It would be interesting to examine the direct amplification processes developed in this research for use with quantitative PCR. This may permit the quantitative assay of a DNA sample concomitantly with its genotype.

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PUBLICATIONS AND PRESENTATIONs

Campitelli L.; Ciccozzi M.; Salemi M.; Taglia F.; Boros S.; Donatelli I.; Rezza G. H5N1 influenza virus evolution: a comparison of different epidemics in birds and humans (1997-2004). J Gen Virol. 2006 Apr;87(Pt 4):955-60.

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Taglia, F.; Fogliatto Mariot, R.; Ghemrawi, M.; and McCord B. Long-reads sequencing Technique and its application to Y-Chromosome as a powerful tool in forensic investigations
The poster presentation was held at 30th International Symposium on Human Identification (ISHI) on September 24th-26th 2019, Palm Springs. I was chosen as one of these four ISHI student Ambassadors and gave another presentation on my research in the form of an interview later broadcasted on ISHI news YouTube channel.

Taglia, F.; Wang, L.; Yan-Hung Yu, E.; Thomas, K.; Gibson-Daw, G.; Bolens, D.; Mariot, R.; Roig, M.; Baud, M.; Ghemrawi, M.; Lothridge, K.; McCord, B. and Lee, S. Accelerating DNA extractions using a microwave toward increasing the Speed and Success of Rapid DNA Analysis. (Poster oral presentation), Virtual 9th Annual Forensic Science Symposium June 1-5, 2020.

Taglia, F.; Wang, L.; Fernandez Tejero, N.; O'Brien, R.; Lothridge, K.; McCord, B. and Lee, S. Increasing the Speed and Efficiency of DNA extractions using a microwave toward increasing the Speed and Success of Rapid DNA Analysis. (Poster) Virtual 73rd Annual Scientific Meeting of the American Academy of Forensic Sciences (AAFS) February 15-19, 2021