Leonard, Raechal. Comparison of Deparaffinization Methods With DNA Quantification. Master of Science (Biomedical Sciences, Forensic Genetics). August 2016. 29 pp., 13 Tables, 12 Figures, 22 References.

Formalin-fixed, paraffin embedded (FFPE) tissue samples are often the only sample type available for testing in pathological and clinical fields. Obtaining usable DNA from FFPE samples is difficult due to the formalin fixation method. Formaldehyde, a large component of formalin, causes DNA-protein cross-linking as well as other issues that must be overcome to obtain useable DNA. Many methods for deparaffinization exist to reduce the issues that arise when working with FFPE samples. This project focuses on comparing two methods of deparaffinization: a method described by Bosso and Al-Mulla and a method used by the University of North Texas Center For Human Identification (UNTCHI), to determine if there is a significant difference in DNA obtained from FFPE samples.

COMPARISON OF DEPARAFFINIZATION METHODS WITH DNA QUANTIFICATION

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center

at Fort Worth

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Raechal Leonard, B.S.,

Fort Worth, Texas

August 2016

ACKNOWLEDGMENTS

Thank you to Ms. Anne-Marie Brun at the Microscopy Core Facility, Department of Cell Biology and Immunology at the University of North Texas Health Science Center for providing all of the samples used in this project.

I would like to thank my major professor, Dr. Joseph Warren, for all his guidance and patience with me through out the whole project. For answering all my questions and helping me with getting all the paperwork I needed done. His help has been integral to the success of not only this project, but the success of me as a student and forensic scientist. I would also like to thank my committee: Dr. Bobby LaRue, Dr. Lisa Hodge, and Dr. Xiangrong Shi for all their input and help with this project. And for their patience with all the delays that occurred.

I would like to thank my friend and fellow FGEN, Katharine Murphy for all her help with editing and for getting me through a lot of tough times. I would not have made it this far without all her support and all our sushi lunch meetings. I would also like to thank my best friend, Megan Edwards for all her support and help, despite her being over 1000 miles away. For always being there when I needed advice or someone to talk to about anything and everything, and for staying up with me on Skype through many late night writing sessions.

Finally, I would like to thank my family for all their support, both emotionally and financially through my entire graduate school career. I would not have had the confidence to pursue this degree without their encouragements and motivating me even when I wanted to give up.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

In pathology and for use in clinical diagnostics, formalin-fixed, paraffin-embedded (FFPE) tissue samples are the most widely used sample type. Many pathological and medical collections around the world contain these sample types. FFPE samples "represent the largest available source of biological materials" [1]. They are often the only type of sample available for diagnostic purposes and molecular and epidemiological studies, as fresh tissue is not always available [1]. These tissue types are easy to store and can be stored for many years, cheaper than other methods, easy to transport, and are suitable for immunohistochemical analyses [1, 2, 3]. However, obtaining deoxyribonucleic acid (DNA) from FFPE samples is difficult.

Aims and Objectives

The project will contribute to the DNA analysis of formalin-fixed, paraffin embedded samples by determining which of two de-paraffinization methods yields the best DNA results. It will primarily focus on comparing two methods, one described by Bosso and Al-Mulla [15] and one used by the University of North Texas Center for Human Identification (UNTCHI) [16].

The objective of this project is to determine which method yields the best results after de-paraffinization. The first aim of this project is to determine if there is a difference between the two methods and the second aim is to determine if there is a significant difference between them. The null hypothesis is the two methods will perform equally and there will be no significant

difference between quantification data obtained. The alternative hypothesis is the method described by Bosso and Al-Mulla [15] will yield increased quantification data as compared to the UNTCHI method [16]. Comparing the two de-paraffinization methods in this research will aide in improving the process of extracting workable DNA from FFPE tissues. Utilizing a technique that better purifies DNA in FFPE tissues will improve the amount of workable DNA from this sample type. This will allow for an improved quality of profiles obtained.

Problem

A difficulty with obtaining usable DNA is that a large component of formalin, which is used in fixation, is formaldehyde. While this does not cause DNA to degrade, it does cause DNA-protein cross-links to occur [1, 4, 5, 7]. Figure 1, in Appendix I, shows the crosslinking that forms on the introduction of formaldehyde to DNA [5]. The cross-link between DNA and proteins must be broken during the extraction process to obtain workable DNA from FFPE samples.

An issue with FFPE samples occurs during fixation. The fixatives solution is often un-buffered during this step [5, 6]. This can result in the pH being very low, sometimes even lower than 1 [5]. Low pH causes DNA to fragment into pieces that are 200 base pairs (bp) or less in size [6]. Fragments of DNA that are 200bp or less can result in larger short-tandem repeats (STRs) dropping out, or not amplifying, during the polymerase chain reaction (PCR) step of DNA analysis [6]. A deparaffinization protocol is usually done before DNA extraction minimize these issues.

There are many different deparaffinization methods available for use, but comparing the different methods to find the best to use is difficult. Often, these methods are not comparable [5].

Nucleic quality depends on multiple factors during pre-fixation, fixation, and post-fixation, causing comparison to be difficult [5]. The pre-fixation factors include tissue type, amount of tissue, and degree of autolysis, which is the "breakdown of all or part of a cell or tissue by self-produced enzymes" [8]; fixation factors include pH, temperature, duration of fixation, and the fixatives used; and the post-fixation factors include temperature and duration of storage [1, 5, 7]. The use of so many different factors is what often leads to conflicting data between studies and can make comparison between methods confusing when deciding which to use [5].

DNA Extraction

Once a deparaffinization protocol is chosen and performed on the samples, DNA extraction is performed. The most commonly used DNA extraction method for FFPE samples is known as Phenol:Chloroform:Isoamyl Alcohol (PCIA)[3, 9]. PCIA is a method that is commonly used in forensic labs and has been the gold standard of DNA for years [9].

In 1869, Friedrich Miescher, a Swiss physician, performed the first ever isolation of DNA [10]. This extraction was crude and did not yield enough DNA for further analysis [10]. The use of phenol in the extraction of nucleic acids (NAs) from mammalian tissues was first described in 1956 by K.S Kirby [11]. Kirby used phenol to extract ribonucleic acids (RNA) from rat liver tissue. The study demonstrated that RNA could be isolated using phenol, sodium acetate, and ethanol [11]. This method separated the solution into two phases, an aqueous (top) phase and an organic (bottom) phase. The location where the two phases meet was known as the interphase [13]. Nucleic acids, such as RNA and DNA, remained in the aqueous phase and the other cellular material, such as proteins and lipids, were precipitated out into the organic phase. Scientists

eventually discovered that phenol alone did not inhibit DNase activity [10]. This prompted the addition of chloroform and isoamyl alcohol to the method [10].

Phenol changed the folding of proteins, causing the hydrophobic (non-polar) inner chains to move outside and the hydrophilic (polar) outer chains to be folded inside [12]. The folding change, or flipping, of the proteins is what allowed for them to be separated out into the organic phase of the extraction. Phenol is less polar than water, so the less polar components would reside in the organic phase [12]. The more polar component, DNA (more polar due to the negatively charged phosphate backbone), would reside in the aqueous phase, which contained a more polar substance [12]. Figure 2, in Appendix I, demonstrated the separation [12]. Chloroform sharpened the interphase between the two phases allowing the aqueous layer to be more accurately collected [12]. Isoamyl alcohol was added to reduce foaming that sometimes occurs when phenol and chloroform are added together [13].

DNA Quantification

After DNA is extracted, it is then quantified. DNA quantification is required in forensics under lab standards. There are multiple commercial kits available for quantification. Real-time PCR, or qPCR, is a method that amplifies DNA while determining quantity present in a sample [18]. A way that qPCR works is through the use of a TaqMan probe. The probe uses a reporter-quencher probe to determine quantity. A reporter-quencher probe is a probe with a reporter dye on the 5' end and a quencher dye on the 3' end [14]. While the probe is intact, the reporter dye does not fluoresce due its proximity to the quencher dye [14]. The probe attaches, or anneals, to a specific, complimentary sequence between the 5' and 3' primers [14]. Once the polymerase (Taq) reaches the probe, it displaces the 5' end of the probe [14]. The displacement results in the

reporter dye being cleaved, allowing the reporter dye to fluoresce, but this only happens if the probe is complementary to the DNA strand [14]. The polymerase continues to travel down the DNA strand, but the probe is not amplified as the 3' end of the probe is blocked [14]. The fluorescence that occurs at the cleaving of the reporter dye is measured and is used to detect the quantity of DNA in the sample. Figures 3-6, in Appendix I, demonstrate the process of qPCR using a TaqMan probe. [14]. The quantity of the DNA helps to determine if there is enough present in a sample to move forward with downstream analysis, such as amplification using PCR and capillary electrophoresis (CE). The amount required for downstream methods depends on the kits and systems used.

DNA Genotyping

Polymerase Chain Reaction (PCR) was developed in the 80s by Kary Mullis and he was awarded a Nobel Prize for PCR in 1993 [19, 20]. PCR was the method of amplifying DNA. Amplification was similar to making photocopies of DNA. Multiple components were required for amplification by PCR. DNA primers, which are small pieces of DNA that are complimentary to the target region of the DNA, were used to flank the region of DNA so that only the target region was amplified during the process [19]. DNA polymerase, usually Taq (Thermis aquaticus), was a required enzyme used to synthesize the new strands of DNA from the template (sample) DNA during extension [19]. Deoxynucleotide triphosphates (dNTPs), which are free floating nucleotides, were incorporated in the synthesizing of the new DNA strands and the sample DNA, which is used as the template for new strands [19]. Figure 7, in Appendix I, demonstrates the PCR process [19].

Capillary Electrophoresis is a technique used separate DNA based on electrophoretic mobility and size. Electrophoretic mobility was defined as “the rate of migration per unit electric field strength of a charged particle in electrophoresis” [21]. Small pieces of DNA moved quicker through the capillary than larger pieces. The capillary instrument contained a detection window and a capture device to determine size of the sample running through the system. Figure 8, in Appendix I, demonstrates the process of capillary electrophoresis.

CHAPTER II

MATERIALS AND METHODS

Sample Collection and Preparation

10 tubes containing 8 to 10 liver tissue samples in paraffin wax were received from Ms. Anne-Marie Brun at the Microscopy Core Facility, Department of Cell Biology and Immunology at the University of North Texas Health Science Center. The samples were divided into three replicates. Each sample was weighed using a Citizen Scale Inc. Cy 360 scale (Citizen®, Piscataway, NJ). The UNTCHI samples were placed in a 2 mL screw-top tube and the Bosso-AL Mulla samples were placed in a 2 mL dolphin tube (BioExpress, Kaysville, UT).

Sample Deparaffinization

UNTCHI Deparaffinization Method

In each replicate, at least 8 samples were weighed and placed into individual, labeled 2 mL screw-top tubes. 1000 µL of Xylene (Sigma, St. Louis, MO) was added to each sample and vortexed. The samples were placed on an incubator for 15 minutes at 56°C (±1°C); the temperature was recorded at the start and end of each incubation period. Pressure from the tubes was released by unscrewing the cap, followed by re-screwing the cap closed. The samples were centrifuged at 16,300xg for 2 minutes. The supernatant was removed and discarded. These steps were repeated once. 1000 µL of 100% ethanol (EtOH; Pharmaco-Aaper, Farmers Branch, TX) was added to the samples and incubated at room temperature, which was measured and

recorded at the start and end of each incubation period, for 15 minutes. The samples were centrifuged at 16,300xg for 2 minutes. The supernatant was removed and discarded. These four steps were repeated once. The remaining EtOH (Pharmaco-Aaper, Farmers Branch, TX) was allowed to evaporate from the samples and the samples were stored in a fridge at 0°C (±1°C); the temperature was recorded at time of storage.

Bosso and Al-Mulla Deparaffinization Method

At least 8 samples were weighed and placed in individual, labeled 2 mL dolphin tubes (BioExpress, Kaysville, UT). Instead of using slides, as described in the protocol, the samples remained in the tubes. The reagents were added and removed from the tubes by pipette. 1000 µL of xylene (Sigma, St. Louis, MO) was added to each sample; enough to submerge each sample completely. The samples were immersed for 10 minutes, and the xylene was removed and discarded. This was repeated twice. 1000 µL of 100% ethanol (EtOH; Pharmaco-Aaper, Farmers Branch, TX) was added to each sample, enough to submerge each one, and the samples were immersed for 10 minutes. The EtOH (Pharmaco-Aaper, Farmers Branch, TX) was removed from the samples and discarded. This was repeated once. 1000 µL of 95% EtOH (UNTHSC Lab stock, Lot #: RPL41116) was added to submerge each sample and the samples were immersed for 10 minutes. The EtOH (UNTHSC Lab stock, Lot #: RPL41116) was removed and discarded. This was repeated once. 1000 µL of 70% EtOH (UNTHSC Lab stock, Lot #: RPL41116) was added to submerge the samples and the samples were immersed for 10 minutes. The EtOH (UNTHSC Lab stock, Lot #: RPL41116) was removed and discarded. 1000 µL of deionized water was added to each sample and each sample was stored in a fridge at 0°C (±1°C); the temperature was recorded at time of storage.

DNA Extraction

Next, all 20 samples were extracted using the Phenol:Chloroform:Isoamyl Alcohol (PCIA) protocol along with a reagent blank. The samples were transferred to new, labeled 2 mL dolphin tubes and 400 µL of Stain Extraction Buffer Working Solution, 10 mL of Stain Extraction Buffer Stock (UNTHSC Lab Lot: 100515FJB) mixed with 60 mg of dithiothreitol (Amresco, Solon, OH), was added. 5 µL of 20 mg/mL proteinase K (UNTHSC Lab Lot: 101915KM) was added and the samples were vortexed and briefly centrifuged. The samples were placed on an incubator at 56°C (±1°C); the temperature was recorded at the start and end of each incubation period. The samples were allowed to incubate over night; the start and end times of each incubation were recorded. The samples were briefly centrifuged and 400 µL of Phenol:Chloroform:Isoamyl Alcohol (25:24:1; Invitrogen by Thermo Fisher Scientific, Carlsbad, CA) was added. The samples were vortexed for approximately 20 seconds and centrifuged at 16,300xg for 3 minutes. The aqueous (top) layer of each sample was transferred to a new, labeled 2 mL dolphin tube. The samples were briefly vortexed to determine if any organic layer was carried over when transferring the aqueous layer; there was no carry over detected for any of the 17-21 samples in each replicate.

PCIA extraction was followed by purification using ethanol precipitation. 1000 µL of 100% EtOH was added to each sample, samples were vortexed gently, and placed in a freezer for 30 minutes. The samples were centrifuged at 16,300xg for 20 minutes. The EtOH was decanted off and 1000 µL of 70% EtOH was added. The samples were centrifuged at 16,300xg for 10 minutes. The majority of the EtOH was removed from the samples using a pipette and the remaining EtOH was allowed to evaporate. 50 µL of deionized water was added to the samples. The samples were allowed to incubate for 2 hours at 56°C (±1°C); the temperature was recorded

at the start and end of each incubation period. The samples were stored in a fridge at 0°C (±1°C); the temperature was recorded at time of storage.

DNA Quantification

After DNA extraction, quantification was done using the Quantifiler® Duo DNA Quantification kit (Applied Biosystems, Foster City, CA). Quantification was done in two batches about a week apart; batch 1 contained samples UNTCHI 6-15 EXT, B-AL 6-15 EXT, and PCIA 2 RB EX, batch 2 contained samples UNTCHI 16-33 EXT, B-AL 16-33, PCIA 3 RB EXT, and PCIA 4 RB EXT. Quantification was done according to manufacturer instructions [14].

DNA Genotyping

10 samples were selected from those with the highest quantification values and were amplified according to manufacturer instructions using the AmpFℓSTR® Identifiler® Plus kit (Applied Biosystems, Foster City, CA). These were then run on a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA) and the data imported into GeneMapper IDX v. 1.4 (Applied Biosystems, Foster City, CA). Profiles were analyzed in GeneMapper IDX (Applied Biosystems, Foster City, CA).

CHAPTER III

RESULTS AND DISCUSSION

Deparaffinization and DNA Quantification

The samples were weighed and documented in each replicate. The purpose of the weighing was to minimize the effect that the amount of sample taken could have on the quantification data. Refer to Tables 1-3 in Appendix II for data. The data obtained from DNA quantification was separated by replicate number and tested for normality using the Shapiro-Wilk Test for normality in RStudio® (RStudio, Boston, MA), refer to Table 4 in Appendix II for quantification data. The test will indicate whether to use a parametric (for normal data) or a non-parametric (for non-normal data) test for further statistical analysis. An α-value of 0.05 was used to determine significance. If the p-value was greater than the α-value (0.05), it would indicate the data was normal. Two of the data sets (quant 1 and quant 2) were not normal; whereas quant 3 was normal, refer to Table 5 in Appendix II for results. The difference in the results of normality could be due to the presence of inhibitors in the samples. FFPE components and phenol are known PCR inhibitors and can skew the quantification results in qPCR reactions. Formalin, the fixative, causes DNA-protein cross-linking and this cross-linking hinders the polymerase activity during PCR as the polymerase would be unable to move through the cross-linked area. Phenol, when not fully removed, will adhere to enzymes in the reaction, preventing amplification from occurring.

Based on the results of the Shapiro-Wilk test, a Wilcox rank sum test was used to

determine the significance, if any, between the quantification data for the UNTCHI method and the Bosso-Al Mulla method for all three replicates. The test is a non-parametric test and is appropriate for this type of data. An α-value of 0.05 was used to determine significance. If the p-value was less than the α-value (0.05), it would indicate a significant difference existed between the two methods. There were no significant differences observed from the test, as all p-values were more than 0.05, refer to Table 6 in Appendix II for results. A Welch Two Sample T-Test was also used to determine significance. The test is a parametric test for significance and was appropriate for this type of data. A t-test was used because one data set was normal, according to the Shapiro-Wilk test, and to confirm the results of the non-parametric test. An α-value of 0.05 was used to determine significance. If the p-value was less than the α-value (0.05), it would indicate a significant difference existed between the two methods. No significance was observed between the methods, refer to Table 7 in Appendix II for results.

The C_T values were also compared. C_T can indicate the presence of PCR inhibitors in a sample, the higher the C_T value, the more likely that an inhibitor is present. FFPE and Phenol:Chloroform:Isoamyl Alcohol are known PCR inhibitors. A Shapiro-Wilk test for normality was performed using RStudio® (RStudio, Boston, MA) and an α-value of 0.05 was used to determine significance. Two replicates, 2 and 3, were normal, but replicate 1 was not, refer to Table 8 in Appendix II for results. Based on the results, a Wilcox rank sum test was used to determine the significance, if any, between the C_T data for the UNTCHI method and the Bosso-Al Mulla method for all three replicates. An α-value of 0.05 was used to determine significance. There was no significant difference observed between the methods, refer to Table 9 in Appendix II for results. Due to two data sets being normal, a Welch Two Sample T-Test was

also used to determine significance. An α-value of 0.05 was used to determine significance. No significance was observed between the methods, refer to Table 10 in Appendix II for results.

DNA Genotyping

Ten samples were chosen from among the highest quantified values for DNA genotyping. The samples were normalized to 0.1 ng, amplified, run on the CE, and imported into GeneMapper IDX v 1.4 (Applied Biosystems, Foster City, CA) for visualization. The amount of loci that were present was noted and compared between the two methods. A locus was counted as present if it had at least one allele peak on the electropherogram. Table 11, in Appendix II, shows which samples were used, the quantification for that sample, and the number of loci present in the profile for each sample. A Shapiro-Wilk test for normality was performed in RStudio® on the data and an α-value of 0.05 was used to determine significance. The data had a normal distribution, refer to Table 12 in Appendix II for results. A Welch Two Sample T-test was performed on the data comparing the amount of loci present between the two methods, as it was the appropriate test for the data. The test was done to determine if one method resulted in a significantly better profile. The more loci that are present, the better the profile. There was a significant difference observed between the UNTCHI method and the Bosso-Al Mulla method in DNA genotyping, refer to Table 13 in Appendix II. The Bosso-Al Mulla samples have significantly less drop out of loci, meaning more loci were amplified, than the UNTCHI samples.

Discussion

While no differences were observed in the quantification data, there was a difference in the profiles obtained. Figures 9 – 12 in Appendix I are the electropherograms for the best and

worst profiles obtained for each method. The electropherograms provide a visual representation of the differences between methods. The samples that were deparaffinized using the Bosso and Al-Mulla method consistently produced better and more complete profiles than the samples deparaffinized using the UNTCHI method.

The Bosso and Al-Mulla samples not only had more loci present, but more of the alleles had larger peak heights. The peaks heights from the Bosso and Al-Mulla samples ranged from 44 relative florescence units (RFUs) to 17232 RFUs and had an average peak height of 1544.38 RFUs. The UNTCHI samples had peak heights that ranged from 95 RFUs to 6189 RFUs and had an average peak height of 208.51 RFUs. The peak heights indicate whether a peak is a true allele or an artifact of the PCR and CE process. Analytical thresholds are set in GeneMapper IDX based on the validation done in the lab. The threshold helps to indicate if an allele is a true one or not. If the peak height of an allele is larger than the threshold calculated then it is most likely are true allele and not an artifact of the PCR and/or CE process. The peak heights can also indicate whether a second, or sister, allele could have dropped out, or failed to amplify during PCR. A stochastic threshold is used to help determine if it is possible for a second allele to have dropped out. The threshold is determined by a validation done in the lab and is higher than the analytical threshold. If a peak reaches or exceeds this threshold, then it is not as likely that a second allele dropped out. If a peak does not reach above the threshold, it is more likely that a second allele dropped out. The Bosso and Al-Mulla samples consistently had more alleles above the stochastic threshold than the UNTCHI samples.

The difference in the profiles may be due to the Bosso and Al-Mulla deparaffinization method undoing the DNA-protein cross-linking that occurs during fixation more efficiently than the UNTCHI method. This could explain why there was no difference in quantification data, but

a significant difference in the profiles. The UNTCHI method requires two incubation steps at 56°C and the Bosso and Al-Mulla method does not. The temperature of the incubation could be a factor in the ability to undo the DNA-protein cross-links. Another possible explanation is that the Bosso and Al-Mulla method was more efficient at removing inhibitors. The Bosso and Al-Mulla method has multiple steps where ethanol is added and removed. This is similar to the ethanol precipitation done to clean, or purify, the DNA extracted from a sample. The extra ethanol steps could result in the DNA obtained being more pure, thus resulting in samples that are less inhibited and more able to be amplified during PCR.

Future experiments could expand the amount of samples used as well as number of replicate experiments. Further research should be done to determine why the Bosso and Al-Mulla method produced significantly better profiles than the UNTCHI method, despite having no significant difference in the amount of DNA obtained from DNA extraction. Determining why the Bosso and Al-Mulla method resulted in better profiles will help to determine if the method is truly better or if it just performed better with these samples. Further research could also look at other tissue types such as muscle, skeletal, connective, or nervous tissue to see if the tissue type effects the profile quality.

CHAPTER IV

CONCLUSIONS

The goal of this research was to compare two deparaffinization methods to determine if one produces significantly better results. Samples were weighed, deparaffinized, extracted and quantified. The data obtained from quantification were compared using both a Welch's two-sample T-test and a Wilcox rank sum test. No significant difference was observed in the amount of DNA obtained between both tests. The C_T values were also compared. There was no significant difference observed between the two methods. Finally, ten samples were chosen for DNA genotyping. There was drop out observed in samples for both methods, but there appeared to be less in the samples that were deparaffinized using the method described by Bosso and Al-Mulla. The amount of loci that had at least one allele present were compared between the methods to determine if there was a significant difference in loci that amplified. A Welch's two-sample t-test was performed on this information. A significant difference was observed in the amount of loci present. The Bosso and Al-Mulla method had significantly more loci present in the profiles obtained than the University of North Texas Center for Human Identification (UNTCHI) method. The Bosso and Al-Mulla samples also had consistently higher peak heights than the UNTCHI method. Based on the data obtained from this study, the Bosso and Al-Mulla method is a more effective deparaffinization method than the method used at the UNTCHI.

APPENDIX I: FIGURES

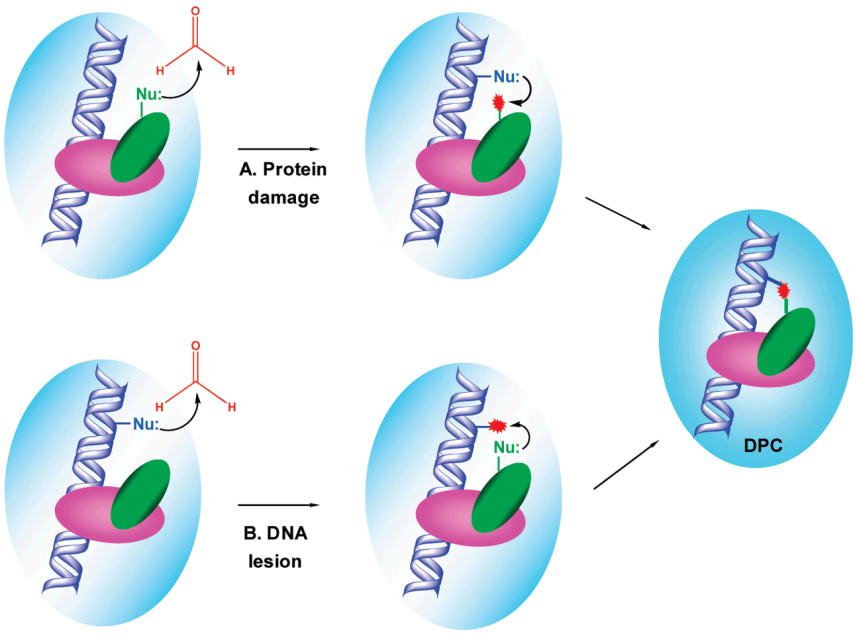

Figure 1: DNA-Protein crosslinking in the presence of formaldehyde [Lu, K., Ye, W., Zhou, L., Collins, L. B., Chen, X., Gold, A., et al. (2010, 02 23). Structural Characterization of Formaldehyde-induced Cross-Links Between Amino Acids and Deoxynucleosides and Their Oligomers. *Journal of American Chemical Society*].

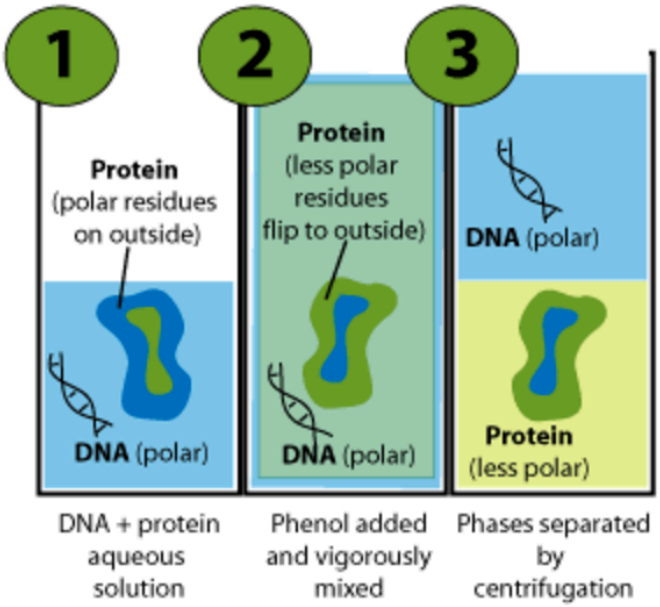

Figure 2: Phenol and protein folding [Oswald, N. (2015, September 8). *The Basics: How Phenol Extraction of DNA Works*. Retrieved March 2, 2016, from BitesizeBIo: http://bitesizebio.com/384/the-basics-how-phenol-extraction-works/]

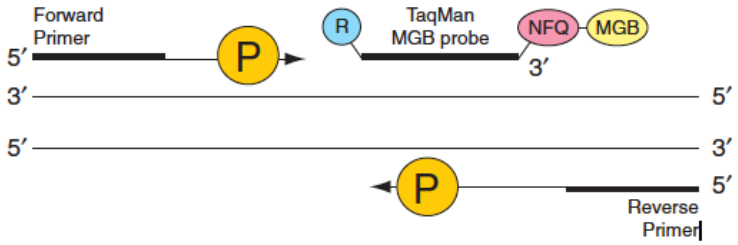

Figure 3: Polymerization [Quantifiler® Duo DNA Quantification Kit User's Manual. Applied Biosystems. Life Technologies Corporation. 2012.]

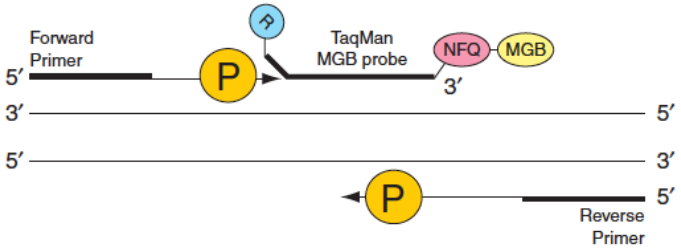

Figure 4: Strand displacement [Quantifiler® Duo DNA Quantification Kit User's Manual. Applied Biosystems. Life Technologies Corporation. 2012.]

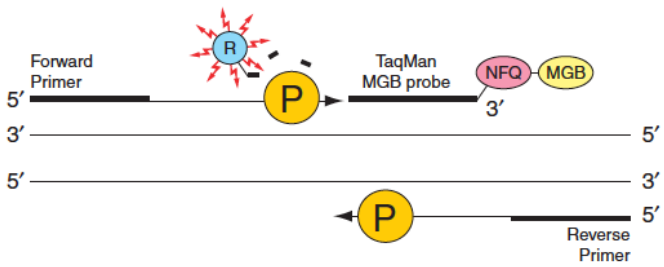

Figure 5: Cleavage of reporter [Quantifiler® Duo DNA Quantification Kit User's Manual. Applied Biosystems. Life Technologies Corporation. 2012.]

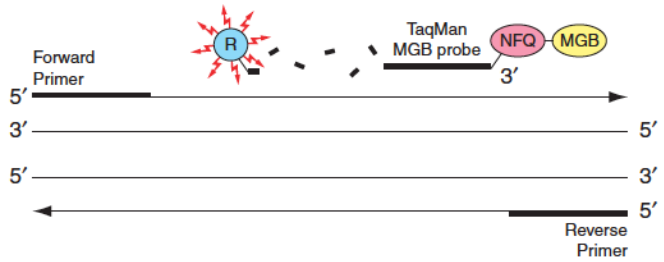

Figure 6: Completion of polymerization [Quantifiler® Duo DNA Quantification Kit User's Manual. Applied Biosystems. Life Technologies Corporation. 2012.].

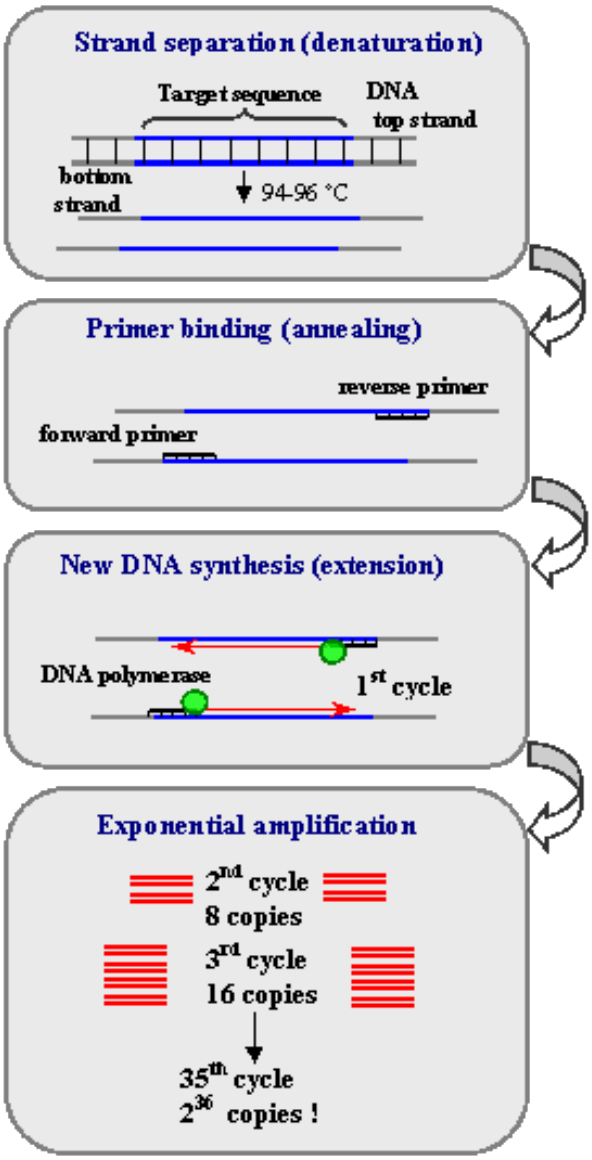

Figure 7: Process of Polymerase Chain Reaction (PCR) [*Polymerase Chain Reaction (PCR)*. Retrieved June 18, 2016, from NCBI: http://www.ncbi.nlm.nih.gov/probe/docs/techpcr/].

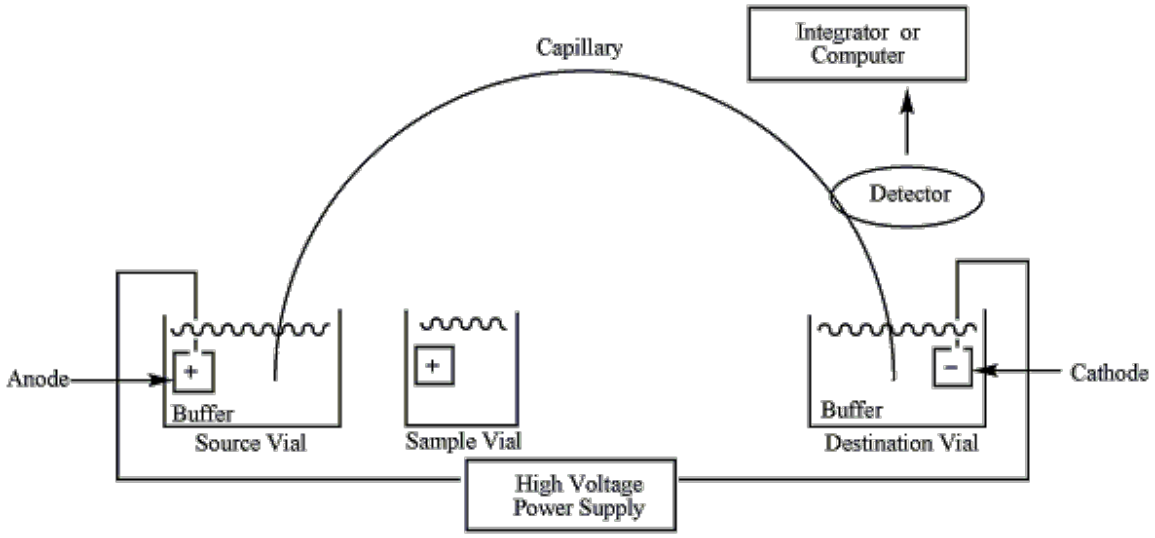

Figure 8: Capillary Electrophoresis [*Capillary Electrophoresis*. Retrieved June 18, 2016, from UC Davis: http://chemwiki.ucdavis.edu/Core/Analytical_Chemistry/Instrumental_Analysis/Capillary_Electrophoresis]

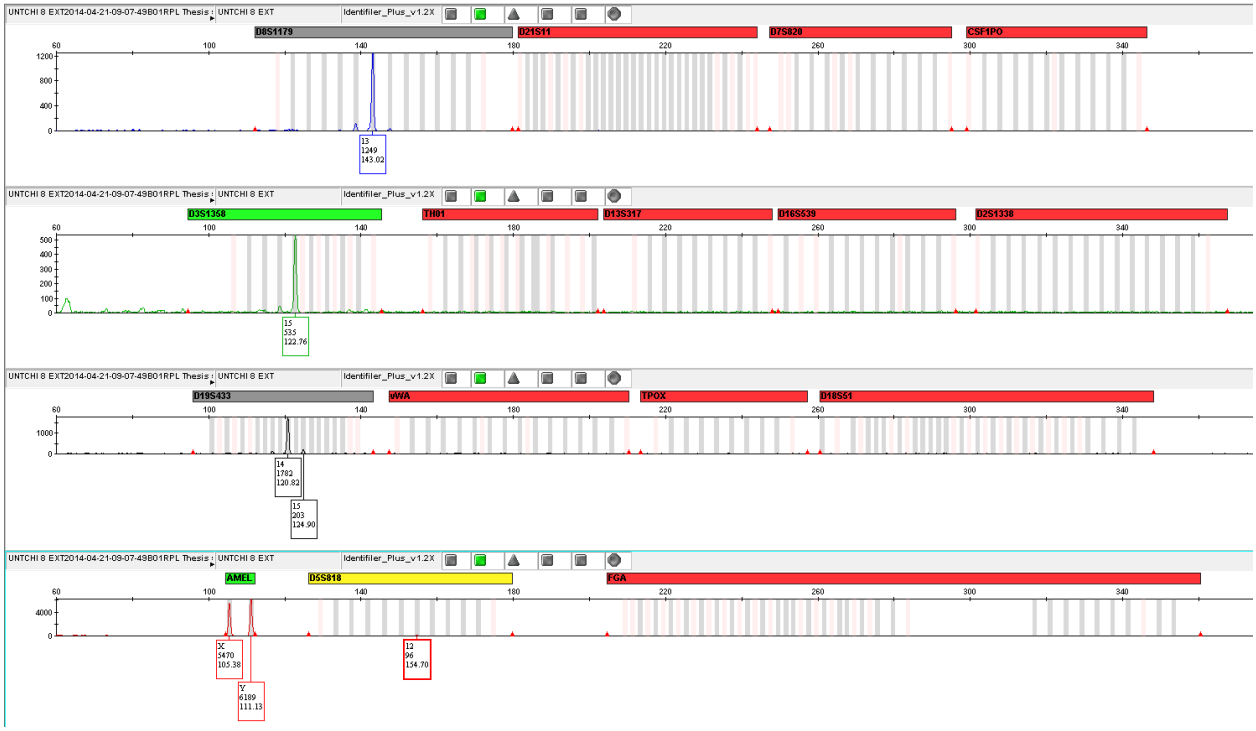

Figure 9: Best profile obtained from a UNTCHI sample, sample UNTCHI 8

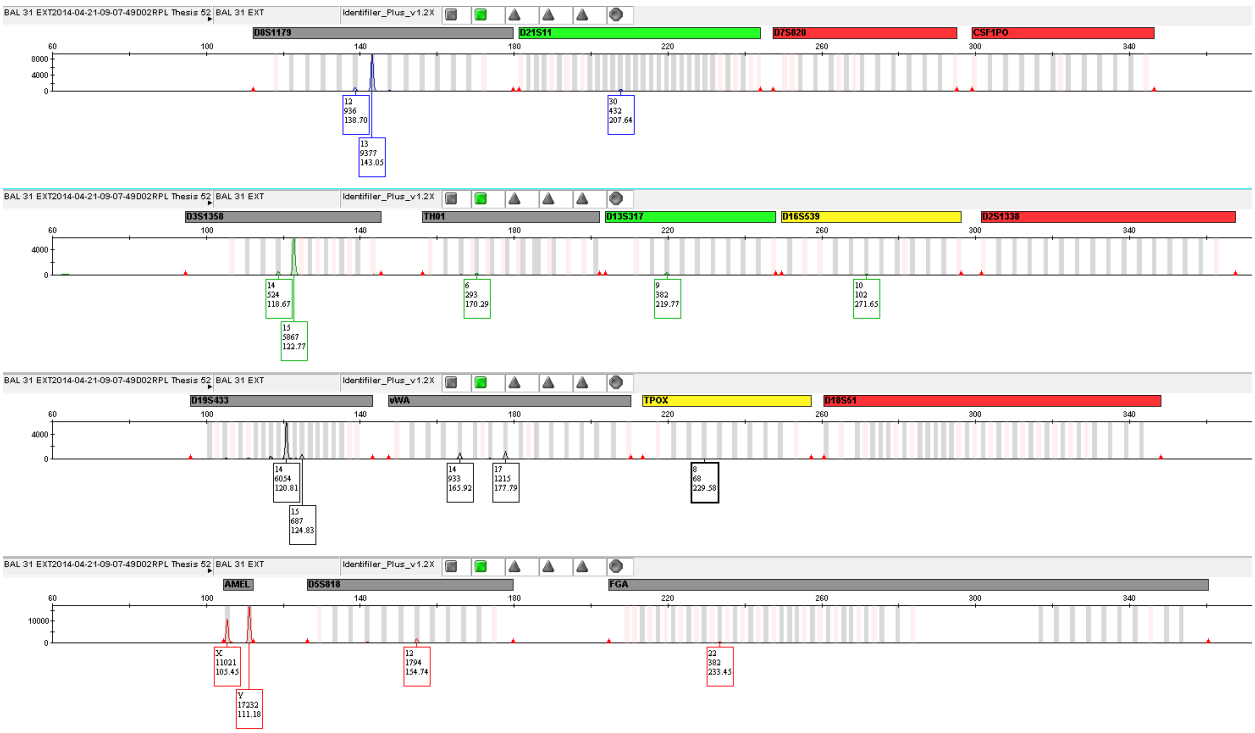

Figure 10: Best profile obtained from a Bosso and Al-Mulla sample, sample BAL 31.

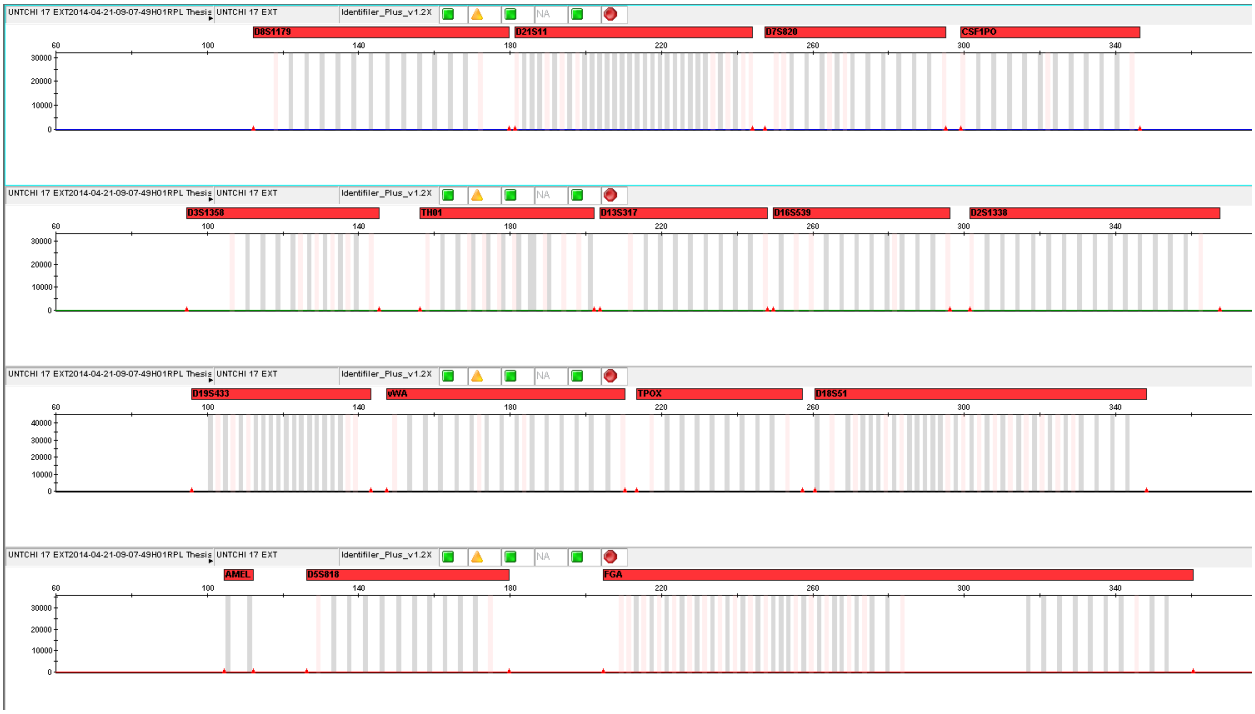

Figure 11: Worst profile obtained from a UNTCHI sample, sample UNTCHI 17

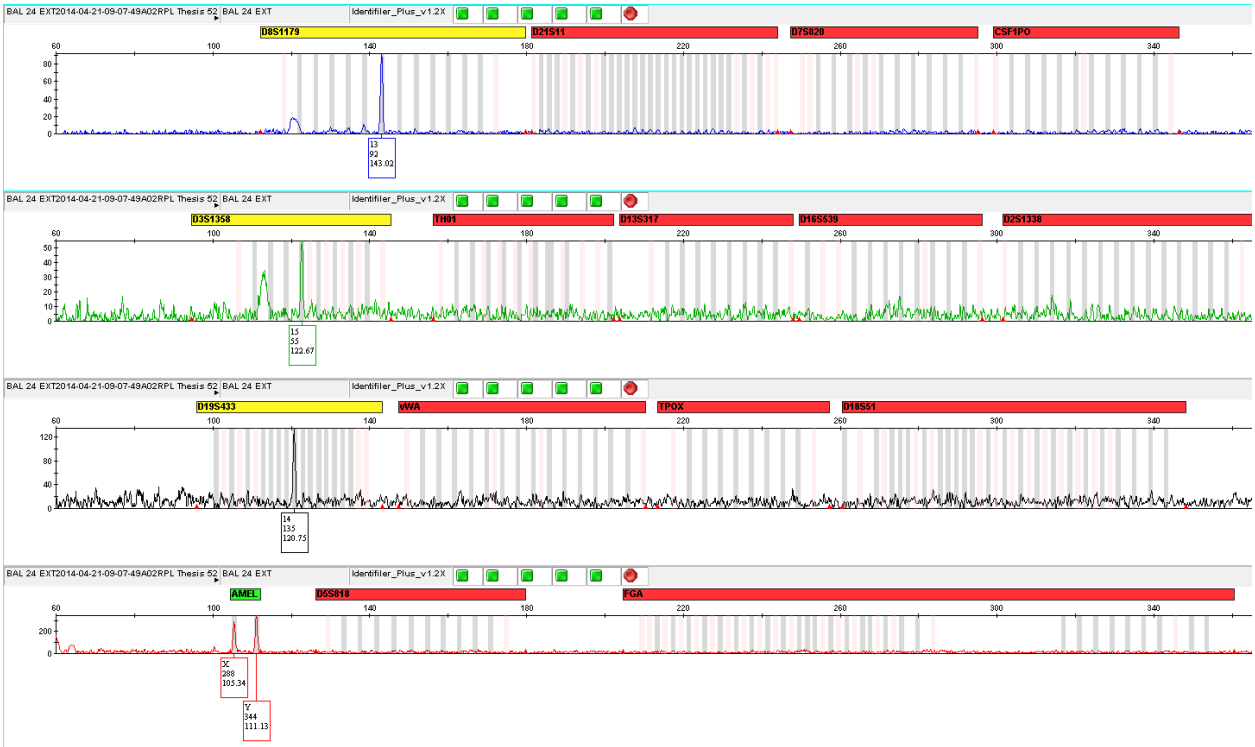

Figure 12: Worst profile obtained from a Bosso and Al-Mulla sample, sample BAL 24

APPENDIX II: TABLES

Table 1: Weights of samples for Experiment 1

Experiment 1			
Sample	Weight (g)	Sample	Weight (g)
UNTCHI 6	0.010	B-AL 6	0.009
UNTCHI 7	0.009	B-AL 7	0.009
UNTCHI 8	0.009	B-AL 8	0.010
UNTCHI 9	0.009	B-AL 9	0.008
UNTCHI 10	0.008	B-AL 10	0.010
UNTCHI 11	0.011	B-AL 11	0.008
UNTCHI 12	0.010	B-AL 12	0.009
UNTCHI 13	0.010	B-AL 13	0.008
UNTCHI 14	0.008	B-AL 14	0.009
UNTCHI 15	0.011	B-AL 15	0.009

Table 2: Weights of samples for Experiment 2

Experiment 2			
Sample	Weight (g)	Sample	Weight (g)
UNTCHI 16	0.010	B-AL 16	0.010
UNTCHI 17	0.011	B-AL 17	0.010
UNTCHI 18	0.009	B-AL 18	0.009
UNTCHI 19	0.011	B-AL 19	0.010
UNTCHI 20	0.010	B-AL 20	0.010
UNTCHI 21	0.009	B-AL 21	0.009
UNTCHI 22	0.009	B-AL 22	0.011
UNTCHI 23	0.011	B-AL 23	0.009
UNTCHI 24	0.009	B-AL 24	0.010
UNTCHI 25	0.009	B-AL 25	0.011

Table 3: Weights of samples for Experiment 3

Experiment 3			
Sample	Weight (g)	Sample	Weight (g)
UNTCHI 26	0.009	B-AL 26	0.011
UNTCHI 27	0.010	B-AL 27	0.009
UNTCHI 28	0.009	B-AL 28	0.011
UNTCHI 29	0.009	B-AL 29	0.009
UNTCHI 30	0.011	B-AL 30	0.009
UNTCHI 31	0.010	B-AL 31	0.010
UNTCHI 32	0.009	B-AL 32	0.010
UNTCHI 33	0.011	B-AL 33	0.009

Table 4: Quantification Data

Sample	Quant 1 (ng/μL)	Sample	Quant 2 (ng/μL)	Sample	Quant 3 (ng/μL)
UNTCHI 6	0.099134125	UNTCHI 16	0.26183933	UNTCHI 26	0.039619375
UNTCHI 7	0.0712393	UNTCHI 17	0.184299648	UNTCHI 27	0.037912566
UNTCHI 8	0.146259263	UNTCHI 18	0.094750911	UNTCHI 28	0.0201074
UNTCHI 9	0.081069209	UNTCHI 19	0.091668092	UNTCHI 29	0.045174371
UNTCHI 10	0.075844206	UNTCHI 20	0.093717538	UNTCHI 30	0.056764975
UNTCHI 11	0.066650294	UNTCHI 21	0.033590004	UNTCHI 31	0.06938038
UNTCHI 12	0.074112035	UNTCHI 22	0.065125056	UNTCHI 32	0.052076187
UNTCHI 13	0.084061705	UNTCHI 23	0.086039431	UNTCHI 33	0.096820369
UNTCHI 14	0.128270507	UNTCHI 24	0.065351881	B-AL 26	0.050671183
UNTCHI 15	0.102638625	UNTCHI 25	0.068350635	B-AL 27	0.0559927
B-AL 6	0.105116859	B-AL 16	0.068541124	B-AL 28	0.068132348
B-AL 7	0.060798436	B-AL 17	0.051468827	B-AL 29	0.075411625
B-AL 8	0.041168816	B-AL 18	0.062006123	B-AL 30	0.017828833
B-AL 9	0.037978005	B-AL 19	0.04811947	B-AL 31	0.094004609
B-AL 10	0.054787274	B-AL 20	0.066281468	B-AL 32	0.060837645
B-AL 11	0.044989087	B-AL 21	0.039529633	B-AL 33	0.11602354
B-AL 12	0.054453943	B-AL 22	0.068767384		
B-AL 13	0.474100411	B-AL 23	0.014586094		
B-AL 14	0.067609482	B-AL 24	0.097216338		
B-AL 15	0.149752706	B-AL 25	0.089572482		

Table 5: Shapiro-Wilk Test for Normality

Shapiro-Wilk Test for Normality		
Replicate	p value	Normal (y/n)
1	8.788E-07	No
2	1.761E-03	No
3	7.839E-01	Yes

$p > 0.05$ is normal

Table 6: Wilcox Rank Sum Test

Wilcox Rank Sum Test		
Replicate	p value	Significant (y/n)
1	0.123	No
2	0.1051	No
3	0.2786	No

$p < 0.05$ is significant

Table 7: Welch Two Sample T-Test

Welch Two Sample T-Test		
Replicate	p value	Significant (y/n)
1	0.7141	No
2	0.07905	No
3	0.2728	No

$p < 0.05$ is significant

Table 8: Shapiro-Wilk Test for C_T Values

Shapiro-Wilk Test for Normality		
Replicate	p value	Normal (y/n)
1	0.03073	No
2	0.1159	Yes
3	0.2899	Yes

$p > 0.05$ is normal

Table 9: Wilcox Rank Sum Test for C_T Values

Wilcox Rank Sum Test: C_T		
Replicate	p value	Significant (y/n)
1	0.123	No
2	0.1051	No
3	0.2786	No

$p < 0.05$ is significant

Table 10: Welch Two Sample T-test for C_T Values

Welch Two Sample T-Test: C_T		
Replicate	p value	Significant (y/n)
1	0.5255	No
2	0.06236	No
3	0.3855	No

$p < 0.05$ is significant

Table 11: Loci Present

Sample	Quant (ng/uL)	Loci Present
BAL 13	0.474100411	4
BAL 15	0.149752706	10
BAL 24	0.097216338	4
BAL 31	0.094004609	12
BAL 33	0.11602354	6
UNTCHI 8	0.146259263	5
UNTCHI 14	0.128270507	0
UNTCHI 16	0.26183933	0
UNTCHI 17	0.184299648	0
UNTCHI 33	0.096820369	2

Table 12: Shapiro-Wilk test for Loci Present

Shapiro-Wilk Test for Normality: Loci Present	
	p value
10 samples chosen	0.1997

$p > 0.05$ is normal

Table 13: Welch Two Sample T-test for Loci Present

Welch Two Sample T-Test: Loci Present		
	p value	Significant (y/n)
BAL vs. UNTCHI	0.01991	Yes

$p < 0.05$ is significant

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