5-2019

DNA Methylation Methods for Donor Age Prediction Using Touch DNA

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DNA METHYLATION METHODS FOR DONOR AGE PREDICTION USING TOUCH DNA

A MASTER'S THESIS

submitted in partial fulfillment

of the requirements for the degree of

MASTER OF SCIENCE IN FORENSIC SCIENCE

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May, 2019

ACKNOWLEDGEMENTS

Sincerest thanks to Professor Timothy Palmbach, Dr. Claire Glynn, and Dr. Lisa McEwen for their support and guidance through this challenging project.

Special thanks to Professor San Pietro for his moral support and troubleshooting ideas; and appreciation to UNH.

ABSTRACT

The International Labor Organization (ILO) estimated over 30 million individuals fall victim to human trafficking each year, of which, 50% are children below the age of 16. In 2012, the ILO reported there to be 168 million child laborers worldwide, with many trafficked into hazardous conditions to manufacture consumer products that are sold in developed countries. This is a modern form of slavery with poor working conditions, no access to education, and low wages. The hidden nature of this crime, however, makes it extremely difficult to identify and locate victims of forced child labor, and thus making it challenging to eradicate.

Children exploited in textile factories typically handle fabrics with bare hands, causing them to shed epithelial cells that contain DNA onto items they are manufacturing. It has been established that touch DNA can be isolated from a variety of substrates, which has the potential to be used to estimate the chronological age of an individual that handled the fabric. DNA methylation is an epigenetic modification which adds a methyl group to the nitrogenous base, cytosine, which can be involved in the regulation of gene expression. Previous research has determined that children have differentially methylated sites in their DNA that can be used as markers to estimate chronological age.

To establish that current procedures could identify DNA from child laborers, touch samples were collected from sixty-seven volunteers within the age range of 0-65 years old on sterile gauze swatches following IRB approval. Total DNA was isolated from the gauze using the DNA Investigator Kit and bisulfite converted using the Qigen EpiTect BC Kit. Samples were quantified using the Qubit® 3 Fluorometer. In addition, some samples were quantified using the Human Quantifiler Kit. Custom primers and TaqMan Probes were designed for several age-associated methylation sites. Two different methylation qPCR kits were attempted for this assay-

the EpiTect MethyLight + ROX Kit and the Methylamp MS-qPCR Fast Kit. Both qPCR assays were unsuccessful at quantifying DNA methylation from touch samples due to the low quantity of original DNA (average 0.092ng/µl). This study makes is clear that touch DNA is extremely difficult to collect in large enough quantities that can be used for downstream analysis.

There is an apparent need for improved touch DNA collection methods. In addition, increased sensitivity of methylation quantification could contribute to optimizing this methodology for future use in chronological age estimation and subsequently identify manufacturers that are exploiting child laborers.

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1 INTRODUCTION

1.1 Human Trafficking

Human trafficking is defined as the illegal trade of human beings, usually across country or state borders, to typically be coerced into forced behaviors. There are 3 types of human trafficking: 1) sexual exploitation; 2) state-imposed forced labor in prisons and military; and 3) forced labor, commonly occurring in manufacturing facilities, such as clothing or electronic factories. It is estimated that there are between 20-30 million victims of human trafficking worldwide, and 50% of victims are below the age of sixteen years old ("Statistics on forced labour, modern slavery and human trafficking," 2017).

1.2 Current Molecular Technique for Identifying Victims of Human Trafficking

DNA typing is a current molecular-based method that has been used for identifying victims of human trafficking in a limited number of cases, and only when comparing a known to unknown profile rather than a human trafficking database inquiry. DNA typing by short tandem repeat (STR) analysis is a common tool used in human identification and is accepted globally in judiciary proceedings. STRs are between 2 and 13 base pairs long and are repeated hundreds of times in a strand of DNA. Because DNA is inherited, each individual acquires unique variations of STRs in different frequencies and lengths. In forensic applications, analysis of STRs measures the number of repeating units encoded in an individual's DNA at 13 different loci (Butler, 2006). The STR loci can then be compared to a reference sample to identify individuals (*2013 Report on Trafficking in Persons*, 2013). The FBI has established a DNA database named CODIS (Combined DNA Index System). CODIS contains DNA characteristics (20 STR loci) that allow

for profile searching through DNA of individuals who have been previously entered into the system from committing prior crimes, missing persons cases, or victims of crimes.

The first limitation of utilizing STRs and CODIS to identify individuals is the need for a reference sample for comparison. If there is only one sample of DNA available, either in CODIS or at a crime scene, then identification cannot be made without the use of a reference. The second limitation of CODIS is that the general public is not included in the database, which prevents a wider range of reference samples that could be used for comparison. The third limitation is that even though CODIS is used as an international database, not all countries are as diligent with adding individual's DNA profiles into the database (Justice, 2014). This negligence could prevent identification of those that are committing crimes or victims associated with crimes. The current approved scope of CODIS does not allow for input for storage of victim's or potential victims of human trafficking. Thus, only local non-network DNA databases are available for this type of use, and thus very limited in their potential.

There are also some limitations in the laboratory protocols for using STRs to identify individuals. The first limitation is that a relatively large quantity of DNA is needed to generate a complete profile from an individual (between 0.5-1ng) (Bruce Budowle, 1998). This can prove to be difficult at crime scenes where trace amounts of bodily fluid may be present. The second limitation is that STRs are relatively expensive to analyze. Each sample from start to finish can cost hundreds of dollars (Thompson et al., 2013).

1.2.1 Forced Child Labor in the Garment Industry

The International Labor Organization (ILO) previously estimated over 1 million children fall victim to human trafficking each year ("Statistics on forced labour, modern slavery and

human trafficking," 2017). The garment industry has been exploiting human trafficking victims for decades. The fashion industry moves quickly, increasing the need for cheaper production costs and increased profit margins. Manufacturers will compete with each other in a “race to the bottom,” meaning that they will lower their labor standards to cut down on production costs so they can secure contracted jobs from companies in need of textile work (Tabb, 2003). The inexpensive production costs attract well-known companies, such as Nike and Walmart, because they are able to make a higher profit on items that cost less to make. Cheap labor is available in many impoverished countries, such as India and Bangladesh, where work is scarce. Children are commonly coerced into forced labor in these clothing production factories with unsafe working environments, no access to education, excessive work hours, and minimal wages.

1.3 Touch DNA

Epithelial cells on the surface of the skin are constantly dying and being shed off to be replaced by new cells. The amount of epithelial cell shedding can increase in response to touching or rubbing rough or abrasive surfaces with the skin. Children that are victims of forced labor usually handle fabrics of different textures with their bare hands, causing them to shed a number of epithelial cells onto the clothes they are producing. Previous studies have validated that DNA can be isolated from shed epithelial cells that have been collected from materials, including fabric, and terming them ‘touch samples’ (Linacre, Pekarek, Swaran, & Tobe, 2010).

1.3.1 Collection Methods

There are several methods currently used by accredited laboratories for the collection of touch DNA samples. These methods include a wet/dry swab technique, in which a sterile cotton swab is moistened with water or sodium dodecyl sulfate (SDS) and rubbed along an area of an evidence item. Immediately following the wet swab, a dry sterile cotton swab is rubbed over the

same area. Two additional methods can also be used for touch DNA collection are mini-taping and gel films, both of which include adhesive strips that can be pressed onto an area to collect touch DNA. DNA can then be extracted from these substrates using a DNA isolation protocol, such as the QIAamp DNA Investigator Kit protocol.

1.4 DNA Methylation

DNA methylation is an epigenetic modification facilitated by an enzyme family called DNA methyltransferases (DNMTs). DNA methylation involves the covalent attachment of a methyl group – comprised of one carbon and three hydrogen atoms – from S-adenyl methionine (SAM) to the fifth carbon in the nitrogenous base, cytosine (forming 5-methylcytosine) (M Okano, 1998). This modification does not change the sequence of nitrogenous bases, but it can result in gene silencing, preventing the synthesis of downstream RNA and proteins (Figure 1) (B. Jin, Li, & Robertson, 2011).

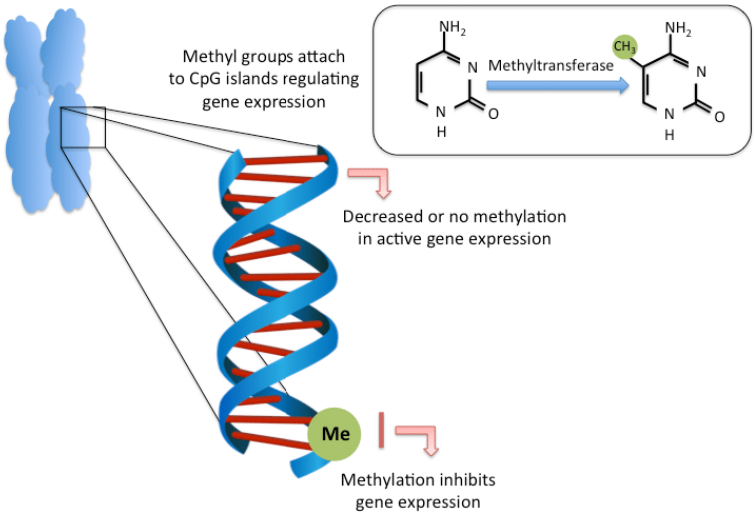


Figure 1 - DNA methylation (Carroll, 2015)

Transcriptional silencing via the DNA methylation landscape is mitotically heritable, meaning it is maintained even when DNA is replicated during mitosis. Because cells follow a

semiconservative replication scheme, the two daughter strands that pair with the original DNA strands have no methyl groups attached. In order to maintain the methylated landscape and prevent any reversal of cell differentiation, an enzyme called DNA methyltransferase 1 (DNMT1) copies the original methylation pattern over to the new daughter strands (Figure 2) (AV Probst, 2009).

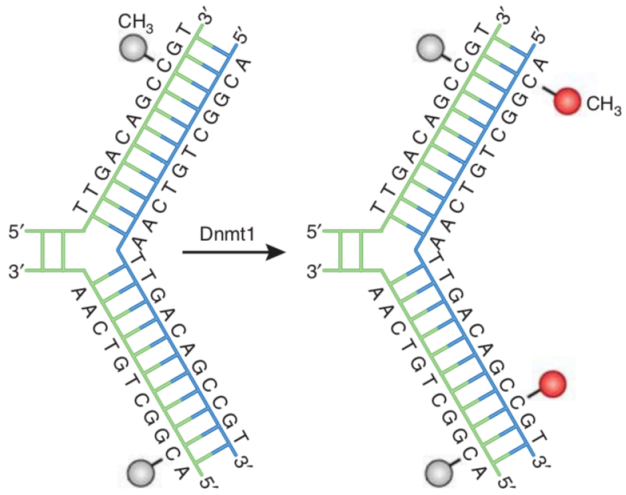


Figure 2 - DNMT1 maintains DNA methylation during replication (Moore, Le, & Fan, 2013)

The most commonly methylated regions of DNA are cytosinephosphate-guanine (CpG) sites (cytosines adjacent to guanine bases). Studies have shown that methylation of CpG sites are not random and that it generally follows some consistent patterns. For example, many active genes have a cluster of CpG sites (termed CpG islands) around the start of their transcription sites, which will be unmethylated in active genes (Bocklandt S, 2011). CpG sites between genes or repetitive DNA sequences are usually methylated (Lister et al., 2009). Expression of genes that have methylated cytosine bases can be reduced by decoder proteins that bind to the methyl groups and prevent transcription factors from binding (J. Li et al., 2018).

The epigenetic landscape is not static, meaning DNA can be demethylated and reactivated in order for changes in gene expression and cell differentiation to occur. A family of proteins called ten-eleven translocation enzymes (TET), are responsible for oxidizing 5-methylcytosine (5mC) to form 5-hydroxymethylcytosine (5hmC). 5hmC can be passively removed during DNA replication of cells undergoing mitosis or actively removed through thymine DNA glycosylase (TDG) base excision and repair of the original DNA strand (Kohli & Zhang, 2013). Covalent modifications, like DNA methylation, allow for flexible transcriptional changes across the genome, while maintaining the original integrity of the sequence of nitrogenous bases.

1.4.1 DNA Methylation Quantification Techniques

Commonly used DNA methylation quantification techniques can be divided into two categories: genome-wide or sequence-specific targeting. Genome-wide scanning provides a low resolution technique for measuring methylation differences in an entire genome across a population of individuals (Assaf Zemach, 2010). Pyrosequencing can be used for both genome-wide methylation quantification and sequence-specific targeting. Pyrosequencing is a sequencing-by-synthesis (SBS) technique that is commonly used for examining genome-wide DNA methylation but can also be used in some instances for gene-specific cases. The preliminary step of Pyrosequencing requires a sodium bisulfite conversion. This technique was developed by two Australian geneticists in the 1990's, who discovered that cytosines that are unmethylated are converted to uracil nitrogenous bases and cytosines that are methylated remain unconverted in the presence of sodium bisulfite (Marianne Frommer, 1992). Once the cytosine nucleotides are converted to uracil, they will be complimentary to thymine bases during downstream sequencing. Methylated cytosines are immune to bisulfite conversion, and will thus

remain complimentary to guanine bases during sequencing (Y. Li & Tollefsbol, 2011). This type of sequencing works by quantifying the incorporation of nucleotides via a light signal produced by the conversion of pyrophosphate (Figure 3) (Gut, 2007). The sequencing will evaluate the ratio of incorporated cytosines versus thymines to determine methylated CpG sites in the DNA (Delaney, Garg, & Yung, 2015).

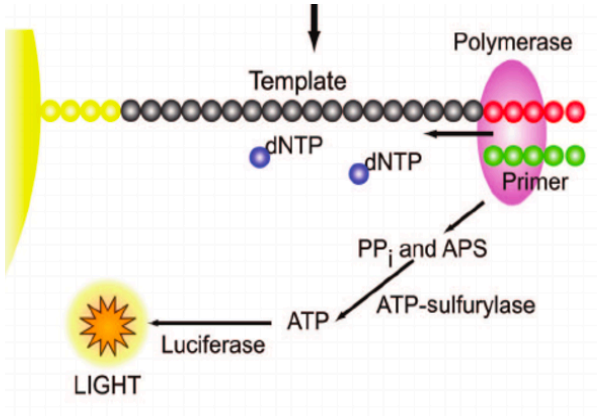


Figure 3 - Pyrosequencing (Voelkerding, Dames, & Durtschi, 2009)

Although Pyrosequencing is considered the most sensitive method for detecting and quantifying DNA methylation, it is also expensive. Pyrosequencing also requires high concentrations of DNA in addition to specialized instrumentation, not common in most forensic laboratories.

Another technique that can be used for genome-wide profiling of DNA methylation is the HumanMethylation 450 BeadChip. This approach utilizes microarray technology, which is essentially a grid of DNA probes attached to a surface that are of known sequence and may be complimentary to genomic bisulfite converted DNA. The probes for sequencing are attached to one of two bead types, methylated or unmethylated. The bead type that is binds to a complimentary strand, is what is detected by the instrument. So, if a region of DNA is

methylated, after bisulfite conversion, it will be complimentary to the methylated probe on the solid array surface. Once the DNA anneals to a probe, a single nucleotide base labeled with a hapten-dideoxynucleotides (ddCTP, ddGTP, ddATP, and ddUTP). The ddATP and ddUTP nucleotides are labeled with 2,4-dinitophenol (DNP) and ddCTP and ddGTP are labeled with biotin (Marabita et al., 2013). Immunostaining is then performed with two antibodies that selectively bind to the complimentary antibody, resulting in a color change to that region on the chip. The instrument detects the color changes, resulting in quantification of methylated versus unmethylated fragments (Figure 4).

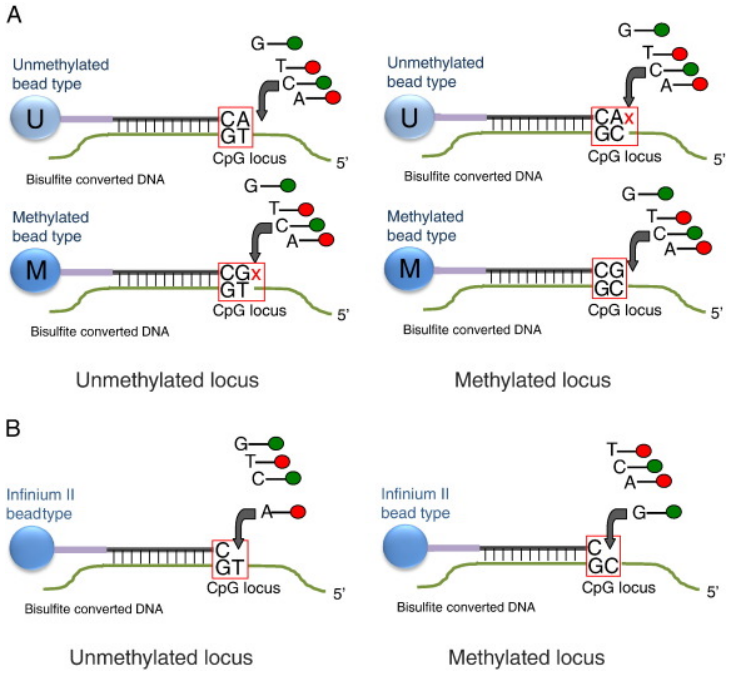


Figure 4 - HumanMethylation 450 BeadChip (Marina Bibikova, 2011)

Real-time quantitative polymerase chain reaction (qPCR) is another DNA methylation quantification technique. Sequence specific primers are complimentary to a flanking region of interest within the genomic DNA. Depending on the system, either a fluorescent intercalating dye that attaches to double stranded DNA, or a reporter dye on a TaqMan Probe is detected by

the instrument to give a real-time readout of the products generated. Most DNA methylation quantification qPCR kits require a bisulfite conversion prior to running the assay. In addition to the low cost, qPCR is also highly sensitive, in most cases, only requiring 50pg of input DNA.

1.4.2 Epigenetics

Genetics is a subgroup of biology that is focused on studying heredity and variation of genes within organisms. Heritable genetic traits can yield phenotypic variation, such as hair and eye color, which is the physical product from a gene sequence encoded by DNA. Epigenetics is the study of modifications to DNA and DNA packaging that are not actually encoded by the DNA sequence itself. Because virtually all cells have the same genetic code for a given individual, epigenetic modifications, such as DNA methylation, can work as a dynamic mechanism for contributing to the regulation of gene expression. These methylation changes are due to both genetic and environmental factors that can change through the course of an individual's lifetime. Differential DNA methylation patterns have been determined to associate with a variety of diseases including neurological illness, cancer, autoimmune and metabolic disorders, as well as aging (Z. Jin & Liu, 2018).

1.4.3 Forensic Epigenetics

Despite the dynamic nature of DNA methylation, the changes across an individual's methylation can create what is known as an epigenetic fingerprint (Vidaki & Kayser, 2018). This unique fingerprint could be used in alongside forensic techniques to gain more information about forensic evidence or an individual that could be linked to a crime.

1.4.3.1 Body Fluid Identification

Determining the tissue type origin of a body fluid at a crime scene can be challenging, but it can be useful for reconstructing events as well as linking individuals together and/or to a

scene. Mixture samples make analysis that much more complex and difficult for analysts. Since 2011, researchers have identified 150 potentially tissue-specific CpG markers that could be used to identify common body fluids such as blood, semen, and saliva (Forat et al., 2016).

1.4.3.2 Differentiation of Monozygotic Twins

Differentiating monozygotic twins from one another is also a challenge for the forensic DNA identification methodology currently in place. Because identical twins share almost exact genomes, it can be very difficult to distinguish them from each other. Because DNA methylation is controlled by both genetics and environmental factors, monozygotic twins commonly have variation in their epigenetic fingerprint, especially in adults (Fraga et al., 2005). One example being a genome-wide methylation study that identified differences in DNA methylation at six sites capable of differentiating 12 monozygotic pairs of twins (Jong-Lyul Park, 2017).

1.4.3.3 Age Estimation

It has been previously determined that children have detectable CpG regions of DNA that are differentially methylated, producing distinctive patterns in children versus adults (Yi, Xu, Mei, Yang, & Huang, 2014). It is also well-established that DNA methylation has an impact on ageing. Using traces of DNA to estimate an individual's age can be forensically relevant for characterizing an unknown perpetrator. However, all DNA methylation age predictors have been established from samples of large volumes of whole blood or saliva that yield high concentrations of DNA, not typically present at crime scenes.

2 LITERATURE REVIEW

2.1 Human Trafficking

It is estimated that there are between 20-30 million victims of human trafficking worldwide, and 50% of victims are below the age of sixteen years old ("Statistics on forced labour, modern slavery and human trafficking," 2017). The caveat to this estimation is that there are no reliable quantitative methods for accurately tracking and locating victims of human trafficking because of the hidden nature of the crime. First-hand reporting is currently the most reliable way to track victims of human trafficking, but the issue with first-hand reporting is that victims of human trafficking rarely come forward due to fear or shame and are commonly isolated from forms of contact. The general public is also not familiar with how to identify potential victims, and if they are, they may be unsure of how to report it.

2.1.1 Child Forced Labor

The garment industry has a reputation for exploiting child workers in order to reduce production costs. Because of this, millions of children have been coerced into the forced labor human trafficking trade via the garment-making industry for decades. This is a modern form of slavery with poor working conditions and low wages for children. This problem stems from the fact that the fashion industry moves quickly, increasing the need for cheaper production costs. Manufacturers will compete with each other in a “race to the bottom,” meaning that they will lower their labor standards to cut down on production costs so they can secure contracted jobs from companies in need of textile work (Tabb, 2003). Cheap labor is available in many impoverished countries, such as India and Bangladesh, where work is scarce. Children are seen as vulnerable and obedient workers, who are commonly coerced into producing garments in unsafe working conditions for minimal wages (Frank Hagemann, 2002). Forcing children to work is illegal in many countries, however, many manufacturers get away with this crime because it is relatively hidden from society and scarcely reported.

2.2 Limitations of Current Molecular Techniques for Identifying Victims of Human Trafficking

The limitations of STR analysis and the CODIS system are contributing to low the identification rate of individuals involved in human trafficking. In 2012, only 40,000 individuals involved in human trafficking were identified by name, out of an estimated 27 million victims and perpetrators (*2013 Report on Trafficking in Persons*, 2013). It is necessary to improve current identification methods to combat the global issue of human trafficking.

As a result of limiting legislation and policies, general awareness about this option, and lack of victim database STR analysis for identifying victims and perpetrators has not yet been proven to be the most efficient and effective way for cracking down on the prevalence of global human trafficking. Further, at this point the use of DNA analysis to help identify a minor victim has not been realized. A novel method of examining DNA methylation methods to estimate age of those involved in human trafficking could potentially provide additional information about an individual in addition to their STR profile. Estimating an individual's chronological age from DNA, could possibly identify manufacturers that are exploiting child workers in their production line.

2.3 Exploited Child Workers Deposit Touch Samples on Manufactured Items

Children that are victims of forced labor in the textile industries usually handle fabrics or textiles of different textures with their bare hands, causing them to shed a number of epithelial cells onto the clothes they are producing. Moreover, any items that is produced with child labor may be a source of epithelial cells. Epithelial cells on the surface of the skin are constantly dying and being shed off to be replaced by new cells. The amount of shedding can increase in response

to touching or rubbing rough or abrasive surfaces with uncovered skin (Daly, Murphy, & McDermott, 2012). Previous studies have validated that DNA can be isolated from shed epithelial cells from materials, including fabric, terming them 'touch samples' (Linacre et al., 2010). In addition, it has been well established that quantifiable amounts of DNA can be collected to be used for downstream analysis including DNA profiling. (Goray, Mitchell, & van Oorschot, 2010).

2.4 DNA Methylation for Estimating Age of Individuals

Development is one biological process where the dynamic transcriptional changes caused by DNA methylation are crucial (J. Li et al., 2018). It has been documented that DNA methylation at CpG sites can change during the course of development and across the life course, which can be detected via various methylation quantification techniques (Johnson et al., 2012). Previous methylation studies have determined that children have detectable CpG regions of DNA that are differentially methylated, producing distinctive patterns in children versus adults (Yi et al., 2014). These known age-associated DNA methylation patterns have the potential to be used as markers to estimate the chronological age of a donor.

There have been several research papers published recently that have examined the differences in DNA methylation across individuals of varying age groups. Zubakov et al. studied DNA methylation in blood samples from individuals between 0-60 years old, and they found that by using just eight age-associated CpG sites and microarray technology, they were able to predict an individual's age with about a 9 year error rate (Zubakov et al., 2010). Garagnani et al. also studied DNA methylation in whole blood samples using the HumanMethylatin 450

BeadChip, and they were among the first to identify two of the strongest age-associated CpG sites, *ELOVL2* and *FHL2* (Garagnani P1, 2012).

Even with the promise of age prediction within a 5-year error rate, Vidaki et al. improved on this by using regression modeling of 23 CpG sites producing an error rate of ± 4.6 years. Due to the development of a neural network learning model, the researchers were able to further increase the performance accuracy to ± 3.3 years (Vidaki et al., 2017).

Instead of using whole blood samples from volunteers, Bocklandt et al. studied DNA methylation differences in saliva samples. With only two CpG sites, they were able to estimate chronological age with an error rate of ± 5.2 years (Bocklandt S, 2011). Hong et al. also used volunteer saliva samples to expand previous work to include 7 age-associated CpG markers. Methylation SNaPshot, which is a single-base extension technique, was used on 226 saliva samples. This research produced one of the lowest age-prediction error rates of ± 3.1 years (Sae Rom Hong, 2017).

Despite differences in methylation quantification techniques and body fluids used, researchers have identified and validated several age-associated CpG sites that are highly discriminatory, including *ELOVL2*, *FHL2*, and *C1orf132*. The methylation techniques used in the studies above are relatively expensive and require large concentrations of DNA. Furthermore, the above studies were partially made possible due to the fact that whole blood, buccal, and saliva samples have higher concentrations of genomic DNA than touch samples (Linacre et al., 2010). Unlike whole-genome methylation studies, gene-specific methods use methylation-specific primers to initiate amplification of only methylated gene-specific areas of DNA (Herman, Graff, Myöhänen, Nelkin, & Baylin, 1996). Aside from microarrays and Pyrosequencing, there are gene-specific assays that could be more cost effective and efficient for

quantifying DNA methylation from low concentrations of DNA, including the EpiTect Methyl qPCR assay, Methylamp MS-qPCR Fast Kit, and the EpiTect MethyLight + ROX Vial Kit.

The EpiTect Methyl qPCR Assay is becoming a a popular option for gene-specific methylation quantification. This assay first uses a methylation-specific restriction digestion to cut the DNA at methylated CpG sites. The digested DNA is then used in a downstream qPCR assay with gene-specific primers to quantify regions of methylated versus un-methylated DNA (Mawlood, Dennany, Watson, & Pickard, 2016). The EpiTect Methyl qPCR assay is more cost effective than Pyrosequencing because it only requires a qPCR instrument, which is commonly found in most DNA laboratories. However, this quantification technique requires large concentrations of DNA (1µg minimum) that are usually obtained from bodily fluids, such as blood. The high concentration of DNA needed to effectively perform the EpiTect Methyl qPCR assay cannot be obtained from touch samples, therefore, other assays will need to be utilized for the quantification of methylation of DNA concentrations below 1µg.

There are two additional methods that could be used to quantify DNA methylation from low concentrations of DNA; which would be in line with concentrations that are isolated from touch DNA samples. The first method is the Methylamp MS-qPCR Fast Kit. This kit requires a sodium bisulfite conversion, just like Pyrosequencing, but then the DNA is incorporated into the assay kit with gene-specific primers for the qPCR run. This reaction only requires a minimum of 50pg of DNA for quantification and costs less than two hundred dollars for one hundred reactions (Minning et al., 2014). The second method is the EpiTect MethyLight + ROX Vial Kit. This method also requires pretreatment of the isolated DNA with sodium bisulfite conversion. Methylation-specific primers and TaqMan probes are also required to amplify targeted DNA during the qPCR reaction. Both of these methods have been demonstrated to be efficient at

quantifying DNA methylation in tissue culture samples, which contain minimal concentrations of DNA, similar to that of touch samples (Minning et al., 2014; van Dijk, Visser, Posthuma, Poutsma, & Oudejans, 2012).

2.5 Research Aims

Due to the concealed manner of human trafficking, it is essentially impossible to currently estimate the exact number of child victims, or identify those involved without first-hand reporting, hence the need for an additional technique that could potentially be used to identify manufacturers that are exploiting child laborers. DNA collected from touch samples on manufactured materials, which are likely to be areas touched by children, but protected from post production incidental handling, could potentially be used for identifying manufacturers that are exploiting child laborers. To date, no one has explored using DNA methylation detection techniques on touch samples to estimate the chronological age of the donor. Therefore, this project is aimed at addressing the following research questions:

1. Can enough DNA be isolated from touch samples to be used for downstream applications?
2. Can the isolated DNA be used to quantify DNA methylation?
3. Can qPCR DNA methylation quantification techniques be used to determine differences in methylation between children and adults?

3 CHAPTER III: MATERIALS AND METHODS

3.1 Sample collection

This study received formal approval from the Institutional Review Board at the University of New Haven to collect human DNA samples from adults and individuals below the age of 18. All DNA samples were collected from volunteers that provided verbal assent and written informed consent. Volunteers below the age of 18 years old required a legal guardian to be present at the time of collection and provide written informed consent. Once informed consent was obtained, the volunteers were provided with a DNA sample collection kit containing a sterile gauze swatch, a sterile cotton swab, and a small glass microscope slide, and asked to do the following:

1. Wash hands with soap and water. Then, rub the sterile piece of gauze between hands for 15 seconds. Place this piece of gauze back into the plastic bag.
2. Rub the cotton swab on the inside of cheek, on both sides for 15 seconds. Place the swab back into the tube.
3. Make a thumbprint on top of the microscope slide by placing thumb onto it and pressing down firmly. Place the microscope slide back into the plastic bag.

67 samples were collected from volunteers across each chosen age group, under 8, 9-17, and 18-60 years old (See appendix 8.1). These age ranges were chosen because they are representative of pre-pubescent, pubescent, and post-pubescent individuals, which have been previously shown to have differential age-associated DNA methylation. Samples were randomly collected from volunteers from affiliates of the UNH Forensic Science Department. All samples were anonymized and stored at -20°C.

3.2 Isolation of Genomic DNA:

The QIAamp DNA Investigator Kit (Qiagen 56504) was used to extract and isolate DNA from all 67 gauze touch samples and 6 buccal swab samples. This kit was chosen for its known ability to extract small quantities of DNA from a variety of substrates. The silica membrane in the spin columns bind to DNA with a high affinity in the presence of salt. The chaotropic salts denature proteins and other hydrophobic cellular material to only leave nucleic acids. Several wash steps remove any impurities and leave only the DNA bound to the membrane. The DNA can then be eluted with buffer that has a low salt concentration.

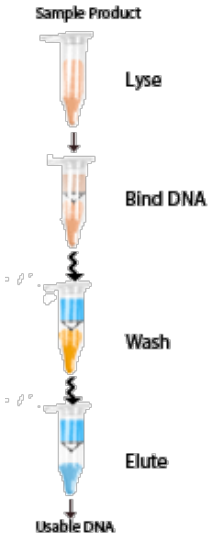


Figure 5 - QIAamp DNA Investigator Kit workflow

The touch samples on the sterile gauze was cut into quarters and placed into separate 1.5mL Eppendorf tubes for the initial cell lysis. A spin basket was used to collect residual eluent from the gauze and then each sample was combined with respective replicates. See appendix 8.2 for detailed extraction protocol. The negative extraction control was sterile gauze that had not

been touched, thus containing no DNA. It has been well documented that buccal swabs yield DNA concentrations that can be reliably used to generate a DNA profile, thus, the positive extraction control was buccal swabs collected from volunteers. Nucleic acid quantitation was performed using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Q32854) following the standard protocol (see appendix 8.5). All eluted samples were stored at -20°C.

3.3 Bisulfite Conversion:

Sodium bisulfite conversion of isolated genomic DNA was performed using the EpiTect Bisulfite Conversion Kit (Qiagen 59104) following the standard protocol (see appendix 8.3). Bisulfite conversion modifies the sequence of DNA by converting non-methylated cytosine nucleotides to uracil. This conversion makes it possible for downstream assays to detect methylated versus non-methylated regions of DNA. Nucleic acid quantitation was performed using the Qubit™ ssDNA Assay Kit (ThermoFisher Q10212) following the standard protocol (see appendix 8.6) in combination with the Qubit® 3 Fluorometer.

3.3.1 Whole Genome

After the isolated genomic DNA was bisulfite converted, the EpiTect Whole Bisulfitome Kit (Qiagen 59203) was used to further amplify fifteen samples (5 samples from each age range of 0-8, 9-17, and over 18). See appendix 8.4 for detailed protocol. Quantitation of the amplified samples was performed using the Qubit™ ssDNA Assay Kit following the standard protocol (see appendix 8.6) in combination with the Qubit® 3 Fluorometer.

3.4 CpG site selection

All CpG targets were selected after a thorough literature search of known age-associated and highly-discriminative CpG sites.

3.4.1 Primer Design

Primer set A (Table 1) was designed for the Methylamp MS-qPCR Assay (EpiGentek P-1028-200). All primers except for the endogenous control, *EF1A*, were designed for bisulfite converted DNA sequences using MethPrimer (Li LC, 2002), a free online software that designs primers based on predicted CpG sites. Primer set B (Table 2) primers and Taqman Probes were designed for the EpiTect MethyLight + ROX Vial Assay (Qiagen 59496).

CpG Site	Forward Primer	Reverse Primer
KLF14	AAGTAGTTTTTTCGGAGCGA	GAAAAATTCGACGACGTC
ELOVL2	TGAGAGGTTTTTGGTTAGTCGT	CTCGAAAAACCCCCGAT
C1orf132	TTATAAGTGTTGATTGATGCGA	TCTAAAAATTCCCCGACGAA
FHL2	TACGGGAGGGGTTATTTATC	TCATCGCGAACTATAAAACG
TRIM59	TTTTGTTTTTCGGGTTGAC	TAACAAAATAAAACCCCGTC
TBX3	ATTTTAATTTGGGAATTGGAGTTTC	AAACCATACTCCTCTTTACTCTCGAC
SST	TGTTTTTTTGGGTTTTTTAGTTTTC	CACCTAAACTATAACCGACTACGCT
TBR1	AGTAAATTTCGGGTTTTAGAATACG	TACCCAATAAACCTTTCCTCTACG
PRPH2	TAGTGAGGTGGTTTTTGTTTATAGC	AACAACCTAAATTTACTCCTAACTCG
CNGA3	TTATTGAATTTTATTTAGGTTTCGG	TTCTAACTATTAAAACCAATCTCGC
KCNAB3	GTTCGTATTATGTTTGTGAAGATTC	TCACCCTCAATAATATACTTTCGAC

Table 1 - Primer Set A

CpG Site	Forward Primer	Reverse Primer	TaqMan Probe	Reporter	Quencher
KLF14	GTTTTTGGGAGAATTCGGG	GAAAACCAACTCGAAACACG	GAAGTTTTACGCGTTTCGTTCGG	FAM	TAMRA

ELOVL2	GGGAGTTCGAGGAAGTCGT	TAACCGTTAAAACCCGAAC	CGTTTGGAGCGGAGAACGGCGTT	FAM	TAMRA
C1orf132	TAAGTGATAGAGTAGAGGAACGGT	CGACAATATAACGACTATCTCCGA	GAGGATTTAGGAGAGTGTAGT	FAM	TAMRA
FHL2	ACGGGAGGGGTTATTTATC	CATCGCGAACTATAAAACGCT	GGTATAAGGAGTGTTTCGTG	FAM	TAMRA
TBX3	GAGAGTAAAGAGGAGTATGGTTTCG	GAAATAATAACGAAACTATAACGTAAATCG	GTTGTTGAGCGGTTTCGGGA	FAM	TAMRA
EF1A	CTGTATTGGATTGCCACACG	GCAGCATCACCAGACTTCAA	AGATTGATCGCCGTTCTGG	FAM	TAMRA

Table 2 - Primer Set B

3.5 Quantification of DNA methylation with qPCR:

There are two different kits that use qPCR-based methods for quantifying DNA methylation. The Methylamp MS- qPCR Fast Kit (EpiGentek cat no. P-1028-200) requires a sodium bisulfite conversion prior to running the assay. The bisulfite conversion was completed by following the protocol in the EpiTect Bisulfite Kit (Qiagen 59104, see sppendix 8.3). Following the bisulfite conversion, the DNA was then used to complete the Methylamp MS-qPCR Fast assay with custom age-associated methylation-specific primers (Table 1). The positive control primer is elongation factor 1 alpha (*EF1A*), which is a house keeping gene that is expressed in all cells that undergo cell division. There are 2 negative controls, 1) the extraction from the sterile gauze containing no DNA 2) a reaction containing no DNA template, yielding no quantification. The positive internal assay control (IAC) was an extracted and bisulfite converted buccal swab. All primers were diluted to a working concentration of 10uM.

Several different qPCR cycles suggested by the manufacturer were used in an attempt to optimize the Methylamp MS-qPCR:

Stage	Temperature (°C)	Time (minutes)	Number of Repeats
1	95	7:00	1
2	95 55 72	0:10 0:10 1:00	45
3	72	1:00	1

Table 3 - Method 1

Stage	Temperature (°C)	Time (minutes)	Number of Repeats
1	95	7:00	1
2	95 55 72	0:15 0:15 1:00	37
3	72	1:00	1

Table 4 - Method 2

Stage	Temperature (°C)	Time (minutes)	Number of Repeats
1	95	7:00	1
2	95 55 72	0:15 0:15 1:00	45
3	72	1:00	1

Table 5 - Method 3

The second kit that uses qPCR-based methods for quantifying DNA methylation is the

EpiTect MethyLight + ROX Vial Kit (Qiagen 59496) with custom primers and TaqMan TAMRA Probes (Table 2). This kit also required a sodium bisulfite conversion of the DNA prior to running the assay. The same sample controls that were used for the Methylamp MS-qPCR kit were repeated for the MethyLight Kit.

Absolute quantification was performed with the Applied Biosystems 7500 Real-Time PCR system.

Stage	Temperature (°C)	Time (minutes)	Number of Repeats
1	95	5:00	1
2	95 60	0:15 1:00	45

Table 6- Method 4

3.6 Quantification of Human DNA:

Quantification of human DNA was completed using the Quantifiler™ Human DNA Quantification Kit (ThermoFisher 434895, see appendix 8.7). This is a qPCR assay that includes a single set of TaqMan probe and primers that are complimentary to human telomerase reverse transcriptase (*hTERT*). A serial dilution is made with the genomic DNA included with the assay, which is then used to generate a standard curve by the qPCR instrument. The standard curve is then used to determine the quantity of human DNA within an unknown sample. 2µL of non-bisulfite converted sample was added to reaction components in respective wells.

3.7 Data Analysis:

The statistical software Genstat was used to perform a one-way ANOVA across touch and buccal sample concentration results from the Human Quantifiler™ qPCR assay.

4 CHAPTER III: RESULTS

4.1 Quantification of Extracted and Bisulfite Converted Samples

All extracted touch samples were quantitated using the Qubit™ dsDNA HS Assay Kit in combination with the Qubit® 3 Fluorometer. The average concentration of isolated DNA from the touch samples was 0.191ng/μL. The average concentration of isolated DNA from control gauze swatches that had not been touched was too low for the Qubit® 3 Fluorometer to detect. All samples were bisulfite converted using the EpiTect Bislufite Conversion Kit. All converted samples were quantitated using the Qubit™ ssDNA Assay Kit in combination with the Qubit® 3 Fluorometer. The average nucleic acid concentration of the converted samples was 6.18ng/μL. The EpiTect Whole Bisulfitome Kit was used to amplify the genomic DNA of 15 bisulfite converted samples (5 samples from each age range 0-8, 9-17, and over 18). All amplified samples were quantitated using the Qubit™ ssDNA Assay Kit in combination with the Qubit® 3 Fluorometer. The average nucleic acid concentration of the amplified samples was 264.4ng/μL.

Kit #	**Extracted DNA concentration from Qubit® (ng/μL)**	**ssDNA concentration from Qubit® (ng/μL)**	**WGA ssDNA concentration from Qubit® (ng/μL)**
1	0.128	1.97	
2	Too low	1.77	
3	0.138	1.68	
4	0.122	11.5	276
5	0.128	1.64	
6	Too low	11.2	
7	Too low	1.94	
9	Too low	9.8	
10	0.118	1.69	
11	Too low	9.28	
12	Too low	8.94	
14	0.103	2.08	
18	0.108	1.96	
20	0.123	7.66	

21	Too low		
23	0.1	9.04	342
24	0.186	8.4	
25	0.176	8.58	480
26	0.145	2.22	
27	Too low		
28	0.119	1.89	
29	0.115	9.44	306
30	0.105	8.4	
32	0.101	7.2	
34	Too low	7.88	
35	0.102	1.94	
40	0.364	8.26	
41	0.107	10.4	200
47	0.102	2.04	
48	0.106	9.46	474
50	0.268	7.52	
51	Too low	6.3	
54	Too low	10.4	
55	Too low	5.96	
56	0.112	9.7	400
57	0.118	8.8	70.4
60	Too low	1.78	
61	Too low		
62	0.156	1.96	
63	0.434		
64	0.23		
65	0.608	2.16	176
66	0.102	1.65	
68	0.49	9.04	440
69	0.17		
71	0.102	11.8	282
72	0.122	9.72	
73	0.17	9.52	193
74	Too low	8.08	
75	Too low	9.26	
76	0.506		
78	0.168	1.84	
79	0.09	2.14	
80	0.18		
81	Too low		
82	0.174		
83	0.246	2.24	91.8
84	0.206		
85	Too low	1.65	

86	0.226	1.91	
87	0.378		
88	0.176	9.66	99.4
89	0.276	2.1	
90	0.38		
91	Too low		
94	Too low	9.68	
101	0.106	10.3	135

Table 6 - Nucleic acid concentrations of extracted and bisulfite converted touch samples

4.2 Quantification of DNA methylation via Methylamp MS-qPCR Fast Kit

One sample from the ≥18 years group (23) was selected for initial optimization of the Methylamp MS- qPCR Fast Kit. All twelve primer pairs (Table 1) were run in triplicate with 4 different sample types, 1) bisulfite converted sample 23 diluted to 1ng/µL, 2) bisulfite converted and whole genome amplified sample 23 diluted to 10ng/µL 3) was an Internal Assay Control (IAC) with non-bisulfite converted DNA run with control primers, 4) was a negative control with *EF1A* primers and no template DNA. Method 1 (Table 1) was used for the qPCR parameters. Results from this initial optimization were inconclusive. The negative control showed evidence of amplification, the non-bisulfite converted samples run with control primers had undetermined cycle threshold (Ct) values, and the sample triplicates were highly variable with no consistency.

Sample Name	**Taget**	**Raw Ct**
23	*KLF14*	41
23	*KLF14*	22.01
23	*KLF14*	2.11
23 WGA	*KLF14*	Undetermined
23 WGA	*KLF14*	2.72
23 WGA	*KLF14*	2.44
23	*PRPH2*	Undetermined

23	*PRPH2*	32.72
23	*PRPH2*	31.23
23 WGA	*PRPH2*	Undetermined
23 WGA	*PRPH2*	27.13
23 WGA	*PRPH2*	28.18
23	*ELOVL2*	Undetermined
23	*ELOVL2*	34.49
23	*ELOVL2*	Undetermined
23 WGA	*ELOVL2*	22.14
23 WGA	*ELOVL2*	21.31
23 WGA	*ELOVL2*	1.8
23	*CNGA3*	Undetermined
23	*CNGA3*	37.91
23	*CNGA3*	36.99
23 WGA	*CNGA3*	Undetermined
23 WGA	*CNGA3*	23.07
23 WGA	*CNGA3*	24.18
23	*C1orf132*	35.23
23	*C1orf132*	40.38
23	*C1orf132*	2.12
23 WGA	*C1orf132*	23.72
23 WGA	*C1orf132*	30.03
23 WGA	*C1orf132*	Undetermined

23	*KCNAB*	Undetermined
23	*KCNAB*	37.42
23	*KCNAB*	Undetermined
23 WGA	*KCNAB*	30.09
23 WGA	*KCNAB*	30.74
23 WGA	*KCNAB*	Undetermined
23	*FHL2*	Undetermined
23	*FHL2*	Undetermined
23	*FHL2*	32.7
23 WGA	*FHL2*	30.46
23 WGA	*FHL2*	29.82
23 WGA	*FHL2*	Undetermined
23	*DLX5*	Undetermined
23	*DLX5*	Undetermined
23	*DLX5*	26.28
23 WGA	*DLX5*	30.76
23 WGA	*DLX5*	Undetermined
23 WGA	*DLX5*	44.61
23	*Trim*	Undetermined
23	*Trim*	Undetermined
23	*Trim*	23.38
23 WGA	*Trim*	19.22
23 WGA	*Trim*	18.87

23 WGA	*Trim*	Undetermined
23	*EF1A*	31.61
23	*EF1A*	Undetermined
23	*EF1A*	29.45
23 WGA	*EF1A*	Undetermined
23 WGA	*EF1A*	27.6
23 WGA	*EF1A*	26.84
23	*TBX3*	Undetermined
23	*TBX3*	1.49
23	*TBX3*	31.31
23 WGA	*TBX3*	28.35
23 WGA	*TBX3*	Undetermined
23 WGA	*TBX3*	29.57
Negative	*EF1A*	Undetermined
Negative	*EF1A*	36.96
Negative	*EF1A*	29.84
Non-bisulfite converted DNA	*EF1A*	Undetermined
Non-bisulfite converted DNA	*EF1A*	Undetermined
Non-bisulfite converted DNA	*EF1A*	25.56
23	*SST*	43.46
23	*SST*	Undetermined
23	*SST*	33.47
23 WGA	*SST*	Undetermined

23 WGA	*SST*	26.12
23 WGA	*SST*	23.39
23	*TBR1*	Undetermined
23	*TBR1*	40.04
23	*TBR1*	Undetermined
23 WGA	*TBR1*	36.59
23 WGA	*TBR1*	Undetermined
23 WGA	*TBR1*	36.92
IAC	*EF1A*	2.58
IAC	*EF1A*	25.57
IAC	*EF1A*	39.8

Table 7 - Raw Ct results (Methylamp MS-qPCR attempt 1)

Sample 23 was used for the second optimization attempt with the Methylamp MS- qPCR Fast Kit. Only primers for *ELOVL2* and *EF1A* from primer set A (table 1) were used. Both primers were run in triplicate with samples 1) bisulfite converted sample 23 diluted to 1ng/µL, 2) bisulfite converted and whole genome amplified sample 23 diluted to 10ng/uL, 3) was non-bisulfite converted DNA run with control primers (IAC), and 4) was a negative control with *EF1A* primers and no template DNA. Method 2 (Table 2) was used for the qPCR parameters, thus increasing the denaturation and annealing times. The quantification results appeared promising with consistent Ct values across replicates. The negative control did show evidence of quantification; however, the Ct values are significantly higher than the other samples, thus it could be likely that the detected fluorescence is background.

Sample Name	**Target**	**Raw Ct**

23	*ELOVL2*	Undetermined
23	*ELOVL2*	34.25
23	*ELOVL2*	36.6
23 WGA	*ELOVL2*	27.7
23 WGA	*ELOVL2*	26.83
23 WGA	*ELOVL2*	28.49
IAC	*EF1A*	22.43
IAC	*EF1A*	23.87
IAC	*EF1A*	24.09
Negative	*EF1A*	37.71
Negative	*EF1A*	Undetermined
Negative	*EF1A*	37.25

Table 8 - Raw Ct results (Methylamp MS-qPCR attempt 2)

To test if the negative control quantification could be eliminated, the same experiment was repeated using the qPCR run parameters from Method 3 (Table 3). This method has the same temperature and time setting as Method 2, but the cycle number is decreased to 37. Results from this experiment were inconclusive. All but four samples had undetermined Ct values and there is no consistency between the sample triplicates Ct values.

Sample Name	**Target**	**Raw Ct**
23	*ELOVL2*	32
23	*ELOVL2*	Undetermined
23	*ELOVL2*	Undetermined
23 WGA	*ELOVL2*	Undetermined

23 WGA	*ELOVL2*	Undetermined
23 WGA	*ELOVL2*	Undetermined
IAC	*EF1A*	17.28
IAC	*EF1A*	Undetermined
IAC	*EF1A*	20.79
Negative	*EF1A*	Undetermined
Negative	*EF1A*	Undetermined
Negative	*EF1A*	31.98

Table 9 - Raw Ct results (Methylamp MS-qPCR attempt 3)

Additional quantities of the Methylamp MS- qPCR Fast Kit with a new lot number were ordered at this time to continue with Method 2, as those qPCR parameters gave the most consistent Ct values across samples. One sample from each age range was selected for preliminary tests of methylation quantitation across all ages; sample 23 (above 18 years old), sample 101 (9-17 years old), and sample 73 (0-8 years old). Only primers for *ELOVL2* and *EF1A* from primer set A (table 1) were used. Both primers were run in triplicate with samples 1) bisulfite converted samples 23, 101, and 73 all diluted to 1ng/μL, 2) bisulfite converted and whole genome amplified samples 23, 101, and 73 all diluted to 10ng/μL, 3) was non-bisulfite converted DNA run with control primers (IAC), and 4) was a negative control with and *EF1A* primers and no template DNA. Results from experiment were inconclusive. The negative control showed evidence of a high quantification, similar to that of the non-bisulfite converted IAC, indicated by Ct values below 20. In addition, the sample triplicates were highly variable with no reliable consistency.

Sample Name	**Target**	**Raw Ct**

23	*ELOVL2*	20.66
23	*ELOVL2*	28.11
23	*ELOVL2*	15.81
23 WGA	*ELOVL2*	21.94
23 WGA	*ELOVL2*	18.9
23 WGA	*ELOVL2*	12.64
IAC	*EF1A*	12.42
IAC	*EF1A*	15.01
IAC	*EF1A*	13.05
Negative	*EF1A*	11.22
Negative	*EF1A*	16.61
Negative	*EF1A*	20.02
101	*ELOVL2*	19.52
101	*ELOVL2*	44.88
101	*ELOVL2*	18.53
101 WGA	*ELOVL2*	21.16
101 WGA	*ELOVL2*	19.93
101 WGA	*ELOVL2*	17.41
73	*ELOVL2*	10.95
73	*ELOVL2*	20.01
73	*ELOVL2*	26.43
73 WGA	*ELOVL2*	17.96
73 WGA	*ELOVL2*	14.39

73 WGA	*ELOVL2*	14.01

Table 10 - Raw Ct results (Methylamp MS-qPCR attempt 4)

Epigentek, the manufacturer of the Methylamp MS- qPCR Fast Kit was contacted for assistance with optimizations. A control experiment was performed using *beta-actin* primers included in the kit to compare to the bisulfite converted sample 23 and *EF1A* primers. All samples were run in triplicate: 1) bisulfite converted sample 23 diluted to 1ng/µL with control *EF1A* primers, 2) was a negative control with *EF1A* primers and no template DNA, 3) was a negative control with *beta-actin* primers and no template DNA, 4) was bisulfite converted sample 23 diluted to 1ng/µL with *beta-actin* primers. The results from this control test were inconclusive. The negative controls showed evidence of high quantification, indicated by the Ct values of 20 and below. In addition, the quantification across all triplicates were highly variable. It was concluded that the Methylamp MS- qPCR Kit did not yield reliable quantification results for the intended assay; thus, a new methodology was used to help eliminate negative control issues and maintain a working stock of touch DNA.

Sample Name	**Target**	**Raw Ct**
23	*EF1A*	22.14
23	*EF1A*	25.59
23	*EF1A*	35.41
Neg EF1A	*EF1A*	13.19
Neg EF1A	*EF1A*	35.96
Neg EF1A	*EF1A*	9.12
Negative	*Beta-actin*	20.17

Negative	*Beta-actin*	20.22
Negative	*Beta-actin*	15.7
23	*Beta-actin*	11.86
23	*Beta-actin*	13.37
23	*Beta-actin*	13.33

Table 11 - Raw Ct results (Methylamp MS-qPCR attempt 5)

4.3 Quantification of DNA methylation via EpiTect MethyLight qPCR + ROX Vial Kit

One sample from each age range was selected for preliminary tests of methylation quantitation across all ages; sample 23 (above 18 years old), sample 101 (9-17 years old), and sample 73 (0-8 years old). Custom primers and TaqMan TAMRA probes for *ELOVL2* and *EF1A* from primer set B (table 2) were used. Both primers with corresponding probes were run in triplicate with samples 1) bisulfite converted samples 23, 101, and 73 all diluted to 1ng/µL with *ELOVL2* primer/probe, 2) bisulfite converted and whole genome amplified sample 23 diluted to 10ng/µL with *ELOVL2* primer/probe, 3) was non-bisulfite converted DNA run with *EF1A* primers (IAC), 4) was bisulfite converted sample 23 diluted to 1ng/µL with *EF1A* primer/probe, and 5) was a negative control with *ELOVL2* primer/probe and no template DNA. Method 4 (table 6) was used for the qPCR run parameters. Results were inconclusive. All samples had undetermined Ct values except for the IAC.

Sample Name	**Target**	**Raw Ct**
23	*EF1A*	Undetermined
23	*EF1A*	Undetermined

23	*EF1A*	Undetermined
23	*ELOVL2*	Undetermined
23	*ELOVL2*	Undetermined
23	*ELOVL2*	Undetermined
101	*ELOVL2*	Undetermined
101	*ELOVL2*	Undetermined
101	*ELOVL2*	Undetermined
73	*ELOVL2*	Undetermined
73	*ELOVL2*	Undetermined
73	*ELOVL2*	Undetermined
Negative	*EF1A*	Undetermined
Negative	*EF1A*	Undetermined
Negative	*EF1A*	Undetermined
IAC	*EF1A*	24.09

Table 12 - Raw Ct results (MethyLight qPCR attempt 1)

The second experiment used a higher DNA concentration of bisulfite converted and whole genome amplified samples 23 and 73 (100ng/µL). Custom primers and TaqMan TAMRA probes for *ELOVL, KLF14, C1orf132, FHL2, TBX3* and *EF1A* from primer set B (table 2) were used. The following samples were run in triplicate: 1) bisulfite converted and whole genome amplified sample 23 diluted to 100ng/µL with *ELOVL2* primer/probe, 2) bisulfite converted and whole genome amplified sample 73 diluted to 100ng/µL with *ELOVL2* primer/probe, 3) bisulfite converted and whole genome amplified sample 23 diluted to 100ng/µL with *EF1A* primer/probe, 4) bisulfite converted and whole genome amplified sample 73 diluted to 100ng/µL with *EF1A*

primer/probe, and 5) was a negative control with *EF1A* primer/probe and no template DNA. The following samples were not done in replicates: 1) bisulfite converted and whole genome amplified sample 23 diluted to 100ng/µL with *KLF14* primer/probe, 2) bisulfite converted and whole genome amplified sample 23 diluted to 100ng/µL with *C1orf132* primer/probe, 3) bisulfite converted and whole genome amplified sample 23 diluted to 100ng/µL with *FHL2* primer/probe, 4) bisulfite converted and whole genome amplified sample 23 diluted to 100ng/µL with *TBX3* primer/probe, and 5) was non-bisulfite converted DNA run with *EF1A* primers (IAC). Method 4 (table 6) was used for the qPCR run parameters. Results were inconclusive. All samples had undetermined Ct values except for the IAC.

Sample Name	**Target**	**Raw Ct**
23 100ng	*ELOVL2*	Undetermined
23 100ng	*ELOVL2*	Undetermined
23 100ng	*ELOVL2*	Undetermined
23 100ng	*EF1A*	Undetermined
23 100ng	*EF1A*	Undetermined
23 100ng	*EF1A*	Undetermined
23 100ng	*KLF14*	Undetermined
23 100ng	*C1orf132*	Undetermined
23 100ng	*FHL2*	Undetermined
23 100ng	*TBX3*	Undetermined
73 100ng	*ELOVL2*	Undetermined
73 100ng	*ELOVL2*	Undetermined

73 100ng	*ELOVL2*	Undetermined
73 100ng	*EF1A*	Undetermined
73 100ng	*EF1A*	Undetermined
73 100ng	*EF1A*	Undetermined
Negative	*EF1A*	Undetermined
Negative	*EF1A*	Undetermined
Negative	*EF1A*	Undetermined
IAC	*EF1A*	21.16

Table 13 - Raw Ct results (MethyLight qPCR attempt 2)

For the next assay, extracted and bisulfite converted buccal swabs of samples 23, 73, and 101 were used. All primers/probes from set B (table 2) were used. The negative control with *EF1A* primer/probe and no template DNA was the only sample run in triplicate. All samples were diluted to 10ng/μL and were run once with the independent primer/probe sets. Method 4 (table 6) was used for the qPCR run parameters. The control primer *EF1A* gave high Ct values (over 35) for all samples. *TBX3* also gave high Ct values (over 35) for buccal sample 23 and 101. There was no quantification in any of the negative controls and the IAC worked as an expected positive control.

Sample Name	**Target**	**Raw Ct**
23 buccal	*ELOVL2*	Undetermined
23 buccal	*KLF4*	Undetermined
23 buccal	*C1orf132*	Undetermined
23 buccal	*FHL2*	Undetermined
23 buccal	*TBX3*	37.64

23 buccal	*EF1A*	33.18
73 buccal	*ELOVL2*	Undetermined
73 buccal	*KLF4*	Undetermined
73 buccal	*C1orf132*	Undetermined
73 buccal	*FHL2*	Undetermined
73 buccal	*TBX3*	Undetermined
73 buccal	*EF1A*	37.44
101 buccal	*ELOVL2*	Undetermined
101 buccal	*KLF4*	Undetermined
101 buccal	*C1orf132*	Undetermined
101 buccal	*FHL2*	Undetermined
101 buccal	*TBX3*	39.08
101 buccal	*EF1A*	36.94
Negative	*EF1A*	Undetermined
Negative	*EF1A*	Undetermined
Negative	*EF1A*	Undetermined
IAC	*EF1A*	21.05

Table 14 - Raw Ct results (MethyLight qPCR attempt 3)

The next experiment was run to complete the sample and primer/probe combinations that gave positive Ct results from the last experiment, in triplicate. The IAC and negative controls were also run in triplicate with control *EF1A* primer/probe.

Sample Name	**Target**	**Raw Ct**
23 buccal	*TBX3*	Undetermined

23 buccal	*TBX3*	Undetermined
23 buccal	*EF1A*	33.15
23 buccal	*EF1A*	33.02
73 buccal	*TBX3*	Undetermined
73 buccal	*TBX3*	Undetermined
73 buccal	*EF1A*	36.09
73 buccal	*EF1A*	35.56
101 buccal	*TBX3*	Undetermined
101 buccal	*TBX3*	Undetermined
101 buccal	*EF1A*	36.04
101 buccal	*EF1A*	36.22
Negative	*EF1A*	Undetermined
Negative	*EF1A*	Undetermined
Negative	*EF1A*	Undetermined
IAC	*EF1A*	21.47
IAC	*EF1A*	21.2
IAC	*EF1A*	21.09

Table 15 - Raw Ct results (MethyLight qPCR attempt 4)

4.4 Quantification of human DNA via Quantifiler™ Kit

Quantification of human DNA was completed using the Quantifiler™ Human DNA Quantification Kit. Results show a statistical difference between the quantities of DNA extracted from touch samples versus buccal swabs.

Sample Name	**DNA Source**	**Quantity (ng/uL)**

23	Touch	1.22E-02
73	Touch	1.38E-03
101	Touch	2.30E-02
23	Buccal	4.65
73	Buccal	2.6
101	Buccal	8.9
65	Touch	1.43E-01
68	Touch	3.20E-01
25	Touch	5.36E-02

Table 16 - Quantification of Human DNA from QuantifilerTM

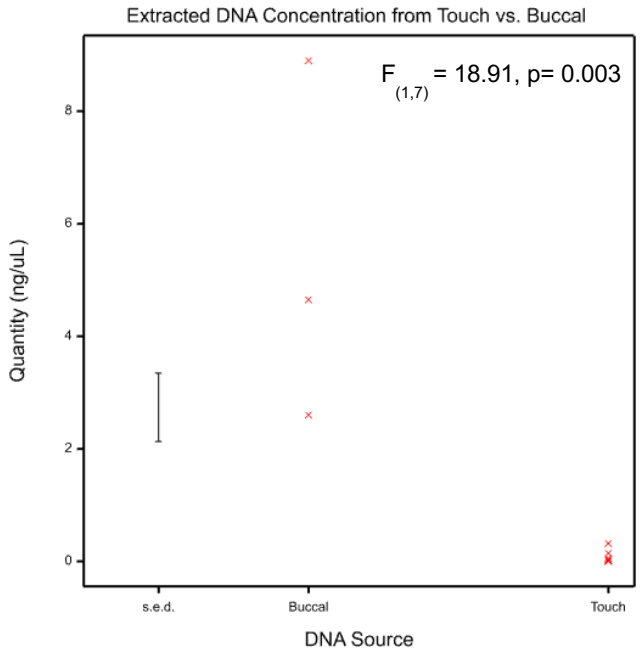


Figure 6 - Recovered touch versus buccal DNA concentrations from QuantifilerTM

5 CHAPTER IV: DISCUSSION

5.1 Methylamp MS- qPCR Fast Kit

The aim of this project was to develop a simple and cost-effective method for estimating the chronological age of an individual from touch-based samples and analyzing DNA methylation via qPCR. One important feature of using qPCR, rather than a Pyrosequencer, is that this type of instrumentation is currently used in modern-day forensic science laboratories. This research showed that the Methylamp MS- qPCR Fast Kit was not specific, yielding low and inconsistent Ct values across replicates, including negative controls. The manufacturer of the kit,

EpiGentek, was contacted for assistance in an attempt to optimize the kit for the qPCR assay; however, low Ct values still resulted for the negative controls even when using the *beta-actin* control primers from the kit. These results led to the hypothesis that the kit could potentially be contaminated or that the manufacturers QA/QC may not be reliable.

5.2 EpiTect MethyLight + ROX Vial Kit

Due to the failure of the Methylamp MS-qPCR Fast Kit, a new kit was purchased from Qiagen called the EpiTect MethyLight + ROX Vial Kit. New primers were designed to sandwich the new TaqMan TAMRA probes for 5 age-related CpG sites and 1 control site (*EF1A*). The MethyLight assay did not yield any Ct values for negative controls or any of the bisulfite converted touch samples. Several optimizations were attempted using touch samples in different concentrations (10ng/µL and 100ng/µL) but all Ct values were undetermined. Buccal samples collected from the volunteers were extracted and bisulfite converted. It has been well documented that touch samples yield the smallest quantities of recovered DNA and buccal swabs result in a higher concentration of DNA (Linacre et al., 2010). All touch samples collected from volunteers had less than 0.2ng/µL of extracted DNA determined by the Qubit®, while the buccal swabs had between 5-13ng/µL. Once converted, the average concentration for touch samples was 6ng/µL and 13ng/µL for buccal swabs. The 15 samples that were amplified using the EpiTect Whole Bisulfitome Kit had DNA concentrations between 90-450ng/µL. The one caveat to these calculated DNA concentrations is that a Qubit® 3 Fluorometer was used, which is not as sensitive and specific as the Human Quantifiler Kit. The MethyLight Kit also does not give any lower range of DNA that it can accommodate for a reaction. The user manual calls for less than 100ng of input template DNA per reaction, but it does not give a lower limit. It was expected that

there would be a difference between the quantification of samples that were bisulfite converted versus samples that were converted and whole genome amplified. The genome amplified samples had a higher concentration of input DNA in the MethyLight assay (10-100ng/µL), and therefore it was expected that there would be lower Ct values for the samples with higher input DNA. However, both bisulfite converted and amplified samples gave undetermined Ct values for the MethyLight assay.

5.3 Touch Samples vs. Buccal Swabs and DNA Quantification

Buccal swab DNA was extracted, and subsequently bisulfite converted in order to assess whether the quantity of touch DNA input was sufficient to be detected by the MethyLight Assay. The buccal swab bisuflite DNA input was 10ng/µL, which was the lower range of what was used for the touch sample input. The assay yielded high Ct values for *TBX* and *EF1A* for samples 23 and 101 in a preliminary experiment. The follow-up experiment was completed in an attempt to repeat the *TBX* and *EF1A* amplification findings in triplicate. *TBX* yielded all undetermined values and *EF1A* was the only gene that was reliably amplified.

These results led to the hypothesis that the Qubit® 3 Fluorometer may not be as sensitive and specific at detecting human DNA quantities from extracted touch samples. In order to test this hypothesis, the Human DNA Quantifiler™ Kit was used to determine concentrations of human DNA. The Quantifiler Kit uses human specific primers in a qPCR reaction to quantify the concentration of human DNA within a given sample. A human cell contains about 6pg of genomic DNA within the nucleus. Historically, 167 cells (about 1ng) worth of genomic DNA is needed for generating a whole profile. The Quantifiler Kit determined that the extracted touch samples had between 0.001-0.3ng of DNA, much less than what is required for even generating a

STR profile in a forensic lab, and thus more than likely too low of a concentration for DNA methylation quantification. The buccal swab DNA concentrations were between 2.6-8.9ng, which is more than enough DNA for generating a STR profile and could be more promising for DNA methylation quantification than the low DNA quantities that were extracted from the touch samples.

5.4 DNA Recovery

There are several potential explanations for the low DNA quantities extracted from the touch samples. One being, gauze is a difficult material to extract DNA from due to the high absorbency and porous nature. Even using a spin basket and centrifuging the sample at maximum speed, it proved difficult to completely dry the gauze, which could have resulted in some loss of DNA. A second possible reason for the low quantities of DNA could be caused by the apoptosis cycle cells undergo when they die. Before epithelial cells are sloughed off to be replaced by new cells, they undergo apoptosis (programmed cell death). During apoptosis, cells release proteins that are responsible for shredding cellular components, including DNA, which could contribute to lower concentrations of recovered DNA (Le Bras & Le Borgne, 2014). Buccal swabs mainly collect cells that are living and non-keratinized, meaning they have intact nuclei that could contribute to higher concentrations of recovered DNA (Cleaton-Jones, 1975). Unlike the touch samples, buccal swabs are also collected with sterile cotton swabs that have less surface area, which could prevent DNA from being left behind on the substrate is it being extracted from.

In the steps prior to estimating the age of a donor from a touch sample, it is crucial to have a DNA collection method that is optimized for the recovery of the highest concentration

possible. Further research has been underway in order to improve current collection methods of low quantity samples. The current standard for collecting touch DNA is either the wet/dry double swab method or mini-taping (which is mostly utilized by forensic laboratories in the UK). More recently there have been additional methods that may prove to be beneficial, including gel films or the M-Vac system. The gel films are an adhesive film, like the mini-tapes but are clear and can be used to visualize cells on the surface prior to extraction using a Trypan Blue stain. The M-Vac system a high-powered wet vacuum that can be used to collect small quantities of DNA from various substrates. Validation studies have demonstrated that enough DNA can be collected from worn articles of clothing to generate a profile, thus also making this technology potentially useful for collecting DNA to be used for methylation studies in order to estimate chronological age (Johannes Hedman, 2015). This method of collection was looked into for this thesis research, however budget constraints prevented access to the necessary equipment.

5.5 Pyrosequencing: an alternative to qPCR-based DNA methylation Assays

A commonly used technique for genome-wide methylation quantification is called Pyrosequencing. Pyrosequencing is a sequencing-by-synthesis (SBS) technique that is commonly used for examining genome-wide DNA methylation but can also be used in some instances for gene-specific cases. Lisa McEwen, PhD., from the Kobor Laboratory at the University of British Columbia developed a pediatric-specific predictor of age using buccal swab DNA methylation of 94 CpG sites, obtained from the Illumina Methylation 450K array, which was able to estimate age of individuals under 20 years old with an absolute median error of less than 0.5 years (McEwen et al., 2019; under review at PNAS). Because this tool uses <100 CpG sites, a sequence-specific technology, such as Pyrosequencing, may be a more cost-effective option for

quantifying methylation to estimate pediatric epigenetic age. This could prove to be extremely useful for donor age estimation of touch DNA samples. The University of New Haven does not currently have access to a pyrosequencer and the samples would be more expensive to send out to a corporation for processing so extracted touch samples were sent to the Kobor Laboratory at the University of British Columbia for pyrosequencing. Time constraints limited the collaboration and no further analysis has been completed with the samples. Another limitation that could be challenging to overcome is that a reliable pyrosequencing run can require about 15ng of input DNA, much larger than any extracted touch sample quantity from this study. It could be valuable to perform a dilution series on some known buccal samples first to determine the lowest possible quantity of input DNA that can be used to still obtain an accurate age estimation.

6 CHAPTER V: CONCLUSION

It should be emphasized that even though there were null results from both of the attempted qPCR-based methylation assays, this study still serves as a valuable starting point for estimating the age of a donor from a touch sample. This study makes it clear that touch DNA is extremely difficult to collect in large enough quantities that can be used for downstream analysis. It is apparent that touch DNA collection methods need to be improved and validated in order to reliably collect high enough concentrations for downstream analysis, including STR profiling and DNA methylation analysis. Furthermore, it would be highly advised to use human-specific quantification, such as the Quantifiler Kit, to determine the quantity of DNA that is extracted. Funding restrictions and availability of some instrumentation limited this research to qPCR-based methods and Qubit® quantification for 95% of the samples. Even with restriction, this research can still provide valuable insight for DNA methylation-based age prediction in touch samples that could potentially be applied to combatting human trafficking worldwide. Overall, this thesis research serves as a valuable starting point for developing future methods for the identification of manufacturers that are exploiting child laborers and bring, what was once a hidden crime, into the forefront of forensic investigation.

7 Future Research

1. Improved touch DNA collection methods
2. Using human specific quantification for touch samples (ie. Quantifiler™ Human DNA Quantification Kit)
3. Pyrosequencing of touch samples
4. Establish a highly age-discriminatory CpG panel that can be used for qPCR assays

8 APPENDICES

8.1 Volunteer Cohort Data

Kit #	Date of Birth	Age of individual on day of collection	Color code
1	3/5/66	52 years 1 month	Green
2	8/7/13	4 years 5 months	Pink
3	9/5/14	3 years 4 months	Pink
4	4/3/96	22 years 5 months	Green
5	11/29/15	2 years 2 months	Pink
6	2/1/06	12 years 2 months	Orange
7	4/5/64	54 years	Green
9	4/19/01	17 years 1 month	Orange
10	2/25/69	49 years	Green
11	1/1/02	16 years 5 months	Orange
12	3/5/03	15 years 3 months	Orange
14	1/8/69	49 years 2 months	Green
18	10/14/11	6 years 4 months	Pink
20	5/27/09	8 years 8 months	Pink
21	3/11/99	19 years 6 months	Green
23	3/8/96	22 years 6 months	Green
24	3/2/04	14 years 3 months	Orange
25	6/28/12	5 years 7 months	Pink
26	4/1/14	3 years 10 months	Pink
27	5/16/09	8 years 8 months	Orange
28	7/1/11	6 years 7 months	Pink
29	7/2/14	3 years 6 months	Pink
30	7/28/16	1 year 6 months	Pink
32	4/13/14	3 years 11 months	Pink
34	4/1/04	14 years 2 months	Orange
35	4/6/16	1 year 9 months	Pink
40	9/29/03	14 years 4 months	Orange
41	4/9/02	16 years 2 months	Orange
47	9/17/14	4 years 4 months	Pink
48	3/17/16	1 year 10 months	Pink
50	10/31/03	14 years 4 months	Orange
51	10/29/04	13 years 3 months	Orange
54	11/9/03	14 years 2 months	Orange
55	4/30/01	16 years 9 months	Orange
56	6/26/05	12 years 7 months	Orange
57	1/4/09	9 years 1 month	Pink
60	6/6/98	19 years 7 months	Green
61	7/17/01	16 years 6 months	Orange
62	4/26/92	25 years 9 months	Green

63	4/28/93	24 years 9 months	Green
64	12/28/93	24 years 1 month	Green
65	9/28/94	23 years 4 months	Green
66	10/26/94	23 years 3 months	Green
68	9/13/07	10 years 4 months	Orange
69	12/3/91	26 years 1 month	Green
71	11/28/00	17 years 6 months	Orange
72	3/9/07	10 years 10 months	Orange
73	7/24/16	1 year 10 months	Pink
74	2/16/01	16 years 11 months	Orange
75	2/16/01	16 years 11 months	Orange
76	8/12/98	19 years 3 months	Green
78	9/4/97	20 years 4 months	Green
79	11/11/97	20 years 2 months	Green
80	2/24/99	18 years 10 months	Green
81	3/18/98	19 years 10 months	Green
82	4/24/98	19 years 9 months	Green
83	4/21/95	22 years 9 months	Green
84	3/26/95	22 years 10 months	Green
85	6/30/95	22 years 7 months	Green
86	12/14/93	24 years 1 month	Green
87	4/15/76	41 years 9 months	Green
88	11/14/96	21 years 10 months	Green
89	2/26/93	24 years 11 months	Green
90	4/16/93	24 years 9 months	Green
91	10/22/07	10 years 3 months	Orange
94	10/22/07	10 years 3 months	Orange
101	1/31/05	13 years 5 months	Orange

8.2 QIAamp DNA Investigator Kit Protocol

Before Starting:

1. ATE Buffer or molecular biology grade water for elution at RT
2. Set 1st heat block to 56C
3. Set 2nd heat block to 70C
4. If buffers AL or ATL have precipitates, dissolve by heating to 70C
5. Be sure that buffers AW1 and AW2 have been diluted with appropriate ethanol *Prior to first use of kit only*

6. Sterilize 2mL tubes if needed
7. Sterilize 1.5mL tubes if needed
8. Sterilize spin columns if needed
9. Incubate aliquot of Buffer ATE at 50C (account for 40uL per sample tube)

For touch samples on gauze:

1. Spread gauze out onto clean kimwipe. Using sterilized scissors, cut the gauze into quarter pieces.
2. Place the gauze swab into a 2mL tube. Use a sterile micropipette tip if gauze needs to be moved to bottom of the tube.

   For buccal samples on cotton swabs:

   2a. Place bulb of cotton swab into a 2mL tube then continue with step 3.
3. Add and 400uL Buffer ATL and 20uL of ProK for cotton substrates. Mix by pulsing vortex for 10sec.
4. Place 2mL tube in heat block or thermomixer. Incubate for 1hr at 56C (shaking at 900rpm if available. If not available, vortex for 10sec every 10min).
5. Spin down tube to remove drops from inside the lid.
6. Add 400uL Buffer AL and pulse vortex for 15 sec.
7. Place 2mL tube in heat block or thermomixer. Incubate for 10min at 70C (shaking at 900rpm if available. If not available, vortex for 10sec every 10min).
8. Spin down tube to remove drops from inside the lid.
9. Add 200uL of 100% EtOH and pulse vortex for 15 sec.
10. Spin down tube to remove drops from inside the lid.

11. Transfer the lysate and gauze from the 2mL tube to a spin column in a clean 2mL collection tube. Centrifuge at max speed for 3 min to dry out the gauze. Discard the spin column with the dried gauze.
12. Transfer all lysate from step 10 to the QIAamp MiniElute column (in the same 2mL collection tube from step 10). DO NOT wet the rim, close lid, centrifuge at 6000g (8000rpm) for 1 min. Place the QIAamp MiniElute column in a clean 2mL collection tube and discard flow through.
13. Add 500uL Buffer AW1 to the QIAamp MiniElute column. DO NOT wet the rim, close lid, centrifuge at 6000g (8000rpm) for 1 min. Place the QIAamp MiniElute column in a clean 2mL collection tube and discard flow through.
14. Add 700uL Buffer AW2 to the QIAamp MiniElute column. DO NOT wet the rim, close lid, centrifuge at 6000g (8000rpm) for 1 min. Place the QIAamp MiniElute column in a clean 2mL collection tube and discard flow through.
15. Add 700uL of 100% EtOH to the QIAamp MiniElute column. DO NOT wet the rim, close lid, centrifuge at 6000g (8000rpm) for 1 min. Place the QIAamp MiniElute column in a clean 2mL collection tube and discard flow through.
16. Centrifuge as max speed (20,000g; 14,000rpm) for 3 min to dry the membrane.
17. Place the QIAamp MiniElute column in a clean 1.5mL tube. Discard collection tube and flow-through. Open the lid of the column and incubate at RT for 10min or 56C for 3min.
18. Apply 40uL of 50C Buffer ATE to the center of the membrane.
19. Close lid and incubate at RT for 5min. Centrifuge at max speed (20,000g; 14,000rpm) for 1 min.

8.3 EpiTect Bisulfite Conversion Kit Protocol

Bisulfite DNA Conversion

1. Thaw DNA to be used in the bisulfite reactions. Dissolve the required number of aliquots of Bisulfite Mix by adding 800 µl RNase-free water to each aliquot. Vortex until the Bisulfite Mix is completely dissolved. This can take up to 5 min.
2. Prepare the bisulfite reactions in 200 µl PCR tubes according to Table 1 (below), page 18. Add each component in the order listed.

   Note: The combined volume of DNA solution and RNase-free water must

Component	Volume per reaction (µL)
DNA solution (1ng-2µg)	Maximum 20µL*
RNase-free water	Variable*
Bisulfite Mix (dissolved)	85
DNA Protect Buffer	35
Total Volume	140

Table 1- Reaction components

* The combined volume of DNA solution and RNase-free water must total 20 µl.

3. Close the PCR tubes and mix the bisulfite reactions thoroughly. Store the tubes at room temperature (15–25°C).
4. Perform the bisulfite DNA conversion using a thermal cycler. Program the thermal cycler according to Table 2 (below).

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	25 min	60°C
Denaturation	5 min	95°C
Incubation	85 min	60°C
Denaturation	5 min	95°C
Incubation	175 min	60°C
Hold	Indefinite	20°C

Table 2- Thermal Cycler Program

5. Place the PCR tubes containing the bisulfite reactions into the thermal cycler. Start the thermal cycling incubation.

Cleanup of bisulfite converted DNA

6. Once the bisulfite conversion is complete, briefly centrifuge the PCR tubes containing the bisulfite reactions, and then transfer the complete bisulfite reactions to clean 1.5 ml microcentrifuge tubes.
7. Add 560 µl freshly prepared Buffer BL containing 10 µg/ml carrier RNA (see "Things to do before starting", page 16) to each sample. Mix the solutions by vortexing and then centrifuge briefly.
8. Place the necessary number of EpiTect spin columns and collection tubes in a suitable rack. Transfer the entire mixture from each tube in step 7 into the corresponding EpiTect spin column.
9. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.
10. Add 500 µl Buffer BW to each spin column, and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.
11. Add 500 µl Buffer BD to each spin column, and incubate for 15 min at room temperature (15–25°C).
12. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.
13. Add 500 µl Buffer BW to each spin column and centrifuge at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.
14. Repeat step 13 once.
15. Place the spin columns into new 2 ml collection tubes, and centrifuge the spin columns at maximum speed for 1 min to remove any residual liquid.

16. Recommended: Place the spin columns with open lids into clean 1.5 ml microcentrifuge tubes (not provided) and incubate the spin columns for 5 min at 56°C in a heating block.
17. Place the spin columns into clean 1.5 ml microcentrifuge tubes (not provided). Dispense 20 µl Buffer EB onto the center of each membrane. Elute the purified DNA by centrifugation for 1 min at approximately 15,000 x g (12,000 rpm).

    Note: To increase the yield of DNA in the eluate, dispense an additional 20 µl Buffer EB to the center of each membrane, and centrifuge for 1 min at maximum speed.

8.4 EpiTect Whole Bisulfitome Kit

1. Place >50 ng bisulfite converted template DNA in 1–10 µl TE buffer or Buffer EB into a microcentrifuge tube. Adjust the volume to 10 µl using nuclease-free water.
2. Thaw REPLI-g Midi DNA Polymerase on ice. Thaw all other components at room temperature, vortex, then centrifuge briefly.
3. Prepare an EpiTect Amplification Master Mix on ice according to Table 1. Mix and centrifuge briefly.

   Important: Add the EpiTect Amplification Master Mix components in the order listed in Table 1. The EpiTect WBA Reaction Buffer should be vortexed for at least 10 s before use. The EpiTect Amplification Master Mix should be kept on ice and used immediately upon addition of the REPLI-g Midi DNA Polymerase.

Component	**Volume per reaction**
EpiTect WBA Reaction Buffer	29µL
REPLI-g Midi DNA Polymerase	1µL
Total Volume	30µL

Table 1- Master Mix Components

4. Add 30 µl of the EpiTect Amplification Master Mix to 10 µl of bisulfate converted DNA (step 1).

5. Incubate the solution at 28°C for 8 h. Place the reaction tubes into a waterbath or heating block at 28°C. If a thermal cycler is used with a heated lid, the temperature of the lid should be set to 70°C.
6. Inactivate REPLI-g Midi DNA Polymerase by heating the sample for 5 min at 95°C. If the amplified DNA will be quantified using PicoGreen® reagent, please note that the reagent only binds double-stranded DNA efficiently. Therefore, quantify the DNA before proceeding with the 95°C incubation, or remove an aliquot (taken after step 5 and cooled to 4°C) for later quantification. If the DNA was quantified after denaturation at 95°C using PicoGreen, multiply the yield by a factor of 2 to compensate for the use of single-stranded DNA.
7. Store amplified DNA at 4°C for short-term storage or –20°C for long-term storage.

8.5 Qubit™ dsDNA HS Assay Kit Protocol

1. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® dsDNA HS Assay requires 2 standards.
2. Label the tube lids.
3. Prepare the Qubit® working solution by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer. Use a clean plastic tube each time you prepare Qubit® working solution. Do not mix the working solution in a glass container.

   Note: The final volume in each tube must be 200 µL. Each standard tube requires 190 µL of Qubit® working solution, and each sample tube requires anywhere from 180–199 µL. Prepare sufficient Qubit® working solution to accommodate all standards and samples.

4. Add 190 µL of Qubit® working solution to each of the tubes used for standards.
5. Add 10 µL of each Qubit® standard to the appropriate tube, then mix by vortexing
6. Add Qubit® working solution to individual assay tubes so that the final volume in each tube after adding sample is 200 µL.
7. Add each sample to the assay tubes containing the correct volume of Qubit® working solution, then mix by vortexing 2–3 seconds. The final volume in each tube should be 200 µL.
8. Allow all tubes to incubate at room temperature for 2 minutes.
9. On the Home screen of the Qubit® 3.0 Fluorometer, press **DNA**, then select **dsDNA High Sensitivity** as the assay type. The "Read standards" screen is displayed. Press **Read Standards** to proceed.
10. Insert the tube containing Standard #1 into the sample chamber, close the lid, then press **Read standard**. When the reading is complete (~3 seconds), remove Standard #1.
11. Insert the tube containing Standard #2 into the sample chamber, close the lid, then press **Read standard**. When the reading is complete, remove Standard #2.
12. Press **Run samples**.
13. On the assay screen, select the sample volume and units:

    a. Press the + or – buttons on the wheel to select the sample volume added to the assay tube (from 1–20 µL).

    b. From the dropdown menu, select the units for the output sample concentration.

14. Insert a sample tube into the sample chamber, close the lid, then press **Read tube**. When the reading is complete (~3 seconds), remove the sample tube.

    The instrument displays the results on the assay screen. The top value (in large font) is the concentration of the original sample. The bottom value is the dilution concentration.

15. Repeat step 2.6 until all samples have been read.

8.6 Qubit™ ssDNA Assay Kit Protocol

1. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® ssDNA Assay requires 2 standards.
2. Label the tube lids.
3. Prepare the Qubit® working solution by diluting the Qubit® ssDNA Reagent 1:200 in Qubit® ssDNA Buffer. Use a clean plastic tube each time you prepare Qubit® working solution. Do not mix the working solution in a glass container.

   Note: The final volume in each tube must be 200 µL. Each standard tube requires 190 µL of Qubit® working solution, and each sample tube requires anywhere from 180–199 µL. Prepare sufficient Qubit® working solution to accommodate all standards and samples.
4. Add 190 µL of Qubit® working solution to each of the tubes used for standards.
5. Add 10 µL of each Qubit® standard to the appropriate tube, then mix by vortexing
6. Add Qubit® working solution to individual assay tubes so that the final volume in each tube after adding sample is 200 µL.

7. Add each sample to the assay tubes containing the correct volume of Qubit® working solution, then mix by vortexing 2–3 seconds. The final volume in each tube should be 200 µL.
8. Allow all tubes to incubate at room temperature for 2 minutes.
9. On the Home screen of the Qubit® 3.0 Fluorometer, press **DNA**, then select **ssDNA** as the assay type. The "Read standards" screen is displayed. Press **Read Standards** to proceed.
10. Insert the tube containing Standard #1 into the sample chamber, close the lid, then press **Read standard**. When the reading is complete (~3 seconds), remove Standard #1.
11. Insert the tube containing Standard #2 into the sample chamber, close the lid, then press **Read standard**. When the reading is complete, remove Standard #2.
12. Press **Run samples**.
13. On the assay screen, select the sample volume and units:

    a. Press the + or – buttons on the wheel to select the sample volume added to the assay tube (from 1–20 µL).

    b. From the dropdown menu, select the units for the output sample concentration.
14. Insert a sample tube into the sample chamber, close the lid, then press **Read tube**. When the reading is complete (~3 seconds), remove the sample tube.

    The instrument displays the results on the assay screen. The top value (in large font) is the concentration of the original sample. The bottom value is the dilution concentration.
15. Repeat step 2.6 until all samples have been read.

8.7 Quantifiler™ Human DNA Quantification Kit Protocol

1. Label eight microcentrifuge tubes: Std. 1, Std. 2, Std. 3, and so on.

2. Dispense the required amount of diluent ($T_{10}E_{0.1}$ Buffer with or without glycogen) to each tube.

3. Prepare Std. 1:

   a. VortextheQuantifiler™HumanDNAStandard3to5seconds.

   b. Using a new pipette tip, add the calculated amount of Quantifiler™Human DNA Standard to the tube for Std. 1.

   c. Mix the dilution thoroughly.

4. Prepare Std. 2 through 8 shown in Table 1 (below)

Standard	Concentration (ng/µL)	Example Amounts	Minimum Amounts
1	50.000	50 µL 200 ng/µL stock] + 150 µL $T_{10}E_{0.1}$	10 µL 200 ng/µL stock] + 30 µL $T_{10}E_{0.1}$ buffer
2	16.700	50 µL Std. 1] + 100 µL $T_{10}E_{0.1}$	10 µL Std. 1] + 20 µL $T_{10}E_{0.1}$ buffer
3	5.560	50 µL Std. 2] + 100 µL $T_{10}E_{0.1}$	10 µL Std. 2] + 20 µL $T_{10}E_{0.1}$ buffer
4	1.850	50 µL Std. 3] + 100 µL $T_{10}E_{0.1}$	10 µL Std. 3] + 20 µL $T_{10}E_{0.1}$ buffer
5	0.620	50 µL Std. 4] + 100 µL $T_{10}E_{0.1}$	10 µL Std. 4] + 20 µL $T_{10}E_{0.1}$ buffer
6	0.210	50 µL Std. 5] + 100 µL $T_{10}E_{0.1}$	10 µL Std. 5] + 20 µL $T_{10}E_{0.1}$ buffer
7	0.068	50 µL Std. 6] + 100 µL $T_{10}E_{0.1}$	10 µL Std. 6] + 20 µL $T_{10}E_{0.1}$ buffer
8	0.023	50 µL Std. 7] + 100 µL $T_{10}E_{0.1}$	10 µL Std. 7] + 20 µL $T_{10}E_{0.1}$ buffer

Table 1- Standard dilution series

5. Calculate the volume of each component needed using Table 2 (below)

Component	Volume per Reaction (µL)
Quantifiler™ Human Primer Mix	10.5
Quantifiler™ PCR Reaction Mix	12.5

Table 2- Reaction Components

6. Thaw the primer mix, then vortex 3 to 5 seconds and centrifuge briefly before opening the tube.

7. Dispense 23µL of the PCR mix into each reaction well.

8. Add 2µL of sample, standard, or control to the appropriate wells.

9. Seal the reaction plate with the optical adhesive cover.

10. Centrifuge the plate at 300rpm for about 20 seconds in a tabletop centrifuge with plate holders to remove any bubbles.

11. Run plate using the specifications for the particular qPCR instrument available.

8.8 IRB Disposition Form

University of New Haven

IRB Disposition Form

Date: November 27, 2017

To: Professor Palmbach

From: Dr. Alexandria Guzmán, IRB Chair AEG

Proposal Title: Approved 11/9/17

Protocol Number: 2017-072

Review by: Committee: Date of Meeting: 10/26/17
Expedited Procedure _____

The IRB has approved the proposed use of human participants in this project.

__X__ The proposal is approved as revised.

_ ___ The proposal is approved with the following minor stipulations. If you agree to the condition(s) of approval, please sign one copy and return it to the IRB chair at irb@newhaven.edu. If the condition(s) of approval are not clear or are unacceptable to you, please contact the IRB chair at irb@newhaven.edu. When submitting revisions, please add a cover letter indicating the revisions and highlight the changes made in the text.

____ The proposal has not been approved in its current form. The committee advises that the revisions below be made and the application be resubmitted for further IRB review. If the suggestions for resubmission are not clear or are unacceptable to you, please contact the IRB chair.

_____ The proposal has not been approved. Reasons for this decision are provided below.

_____

1

PLEASE NOTE

1. **Approval of the project will expire on _Nov 1, 2018_.** Federal regulations (DHHS) and UNH policy require that you submit a "Continuing Review Form" at that time if continued approval is desired. A copy of the "Continuing Review Form" is attached.

2. If the research procedures are altered from the description in the proposal reviewed, you must submit a "Request for Revision Form" to the IRB.

3. Upon approval of the study, a consent document will be stamped with an expiration date. Only this document may be used when enrolling subjects. Studies extending beyond the expiration date must be submitted for a continuation review. Any changes in the consent form must be approved by the IRB.

4 For projects with multiple data collection points, informed consent must be obtained at EACH data collection session.

5. When participant recruitment and data collection are completed for this project, federal regulation and UNH policy require that you submit a "Research Completion Form" to the IRB. After this form is submitted, if you wish to recruit more participants or collect more data for this project, you must submit a new IRB Application form for review.

5. Should any modifications be made in the approved project, a "Request for Revision" form must be submitted to the IRB.

6. If any adverse event or data breach occurs during participant recruitment or data collection an "Adverse Event/Data Breach" form must be submitted to the IRB.

7. If any problems arise concerning the welfare of subjects in the projects, please contact me (irb@newhaven.edu).

8. It is the responsibility of the Principal Investigator to ensure that any person that joins the research team after initial IRB approval be certified prior to interacting or intervening with human participants or their data.

Best wishes for the successful completion of your research.

Project Expires on
NOV 01 2018
University of New Haven
Institutional Review Board

8.9 IRB Donor Consent Forms

Informed Consent Form - Touch Sample

University of New Haven

Title: DNA methylation methods for donor age prediction in touch DNA samples

Investigator: Professor Timothy Palmbach J.D.

Co-Investigators: Dr. Claire L. Glynn, Emily Neverett, Kendra Jones

Participant's Printed Name: **Date of Birth:**

_____ _____

Parent/guardian must be present during the duration of sample collection for anyone under the age of 17 years old.

Introduction

You are invited to participate in a research study designed to look at differences in DNA of various age groups (children and adults) from touch samples on fabric. Your DNA will be extracted from the donated samples but the testing involved will not obtain any genomic or genetic information relating to your health or disease status. Participating in this study is voluntary, and we urge you to ask any questions of the investigators before committing. Talk to your family and friends and take time to make this decision. By signing this form you indicate that you wish to participate in this study.

What is involved?

If you decide to participate in the study, you will be provided with a sample collection kit and asked to do the following:

1. Please wash your hands with soap and water. Then, rub the piece of fabric between your hands for 15 seconds. Place this piece of fabric back into the plastic bag.

2. Please rub the cotton swab on the inside of your cheek, on both sides for 15 seconds. Place the swab back into the tube.

3. Please make a thumbprint on top of the microscope slide by placing your thumb onto it and pressing down firmly. Place the microscope slide back into the plastic bag.

Between the consent form and demonstration on how to collect your sample, we think your participation will take 5 minutes.

You can stop participating in the study at any time.

Risks

This study involves very rare, minimal risks: Allergic reaction to medical grade gauze and cotton swabs, or potential abrasions to skin from friction between fingers and gauze. This study is not designed to inflict any other psychological, social, economic, employability, or civil liability risks.

Benefits

There is no monetary or direct benefits provided to you for participating in this study; however, this study is aimed to forward scientific investigation so others may benefit in the future from your participation.

Confidentiality

We will take the following steps to keep information about you confidential, and protect it from unauthorized disclosure:

- All consent forms will be kept in a locked filing cabinet/password protected files in the Department of Forensic Science at the University of New Haven only accessible by the departments Research Coordinator, Dr. Claire L. Glynn.

- All consent forms will have color coded tabs that will be used to categorize the chronological age of the participant. There will be no unique identification numbers used to label any samples in order to maintain anonymity. Your samples will never be identified using your name in writing or orally.

- The collected samples, data, and written consent forms will be stored for three years. After three years, all biological materials will be destroyed.

- Any data to be published will not include identifiers of the participants, including their names.

As a Participant:

Participation in this study is voluntary. You have the right to cease participation at any time. Deciding not to participate or leave the study will not result in any penalty or harm your relationship with the University of New Haven or other collaborative universities. The samples collected for this study will not be used for any additional studies that you have not provided written consent for.

Contacts for Questions

If you have any questions regarding participation, unexpected physical or psychological discomforts, or use of your samples at any time, please contact: Emily Neverett at 603-620-5318 or email eneve1@unh.newhaven.edu

If you have any questions or concerns regarding this study or your rights as a research participants and would like to talk to someone other than the researcher(s), contact the chair of the Institutional Review Board at UNH irb@newhaven.edu

Consent of Participant:

By signing this consent form, you indicate your and/or your child's voluntary participation in this study.

_____

Printed Full Name of Participant

_____Date: _____

Signature

If signing for a minor (under the age of 18):

_____

Printed Parent or Guardian Full Name

_____Date: _____

Signature of Parent or Guardian

Researcher Obtaining Consent:

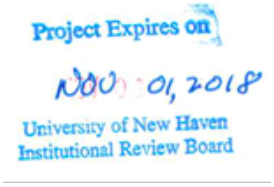
Your signature indicates that you have explained the research to the participant and have answered any questions they may have about the project.

_____

Printed Full Name Researcher

_____Date: _____

Signature



Verbal Assent Form (child over 9 years old) – Touch DNA Sample
University of New Haven

Title: DNA methylation methods for donor age prediction in touch DNA samples
Investigator: Professor Timothy Palmbach J.D.
Co-Investigators: Dr. Claire Glynn, Emily Neverett, Kendra Jones

Parent/guardian must be present during the duration of sample collection for anyone under the age of 17 years old.

Name of Child:_____ **Date of Birth:**_____

Parental Permission on File: ____ yes ____no
(If **"no"** do not proceed with assent or research procedures)

Model Verbal Assent Script for Children (below 17 years old)
Instructions: This model provides suggested language to verbal assent for a child below the age of 17 years old. Child assent will be sought only after written parental informed consent for the child's participation is obtained. -

Hi, my name is [researcher's name]. I am a [teacher/college student at the University of New Haven]. I want to invite you to participate in a research study designed to look at differences in DNA of various age groups from touch samples on fabric. I am trying to learn if I can collect DNA from skin cells left behind on touched objects. Your DNA will be extracted from donated samples but testing involved will give no information relating to your health or disease status. Your participation is voluntary and only your choice. I want to explain what will happen if you decide to participate. I will give you a sample collection kit which will contain the following items; a piece of fabric in a plastic bag, a cotton swab in a tube, and a piece of glass in a plastic bag.

To donate samples to my project, please may I ask you to do three things:

1. *Please wash your hands with soap and water. Then, rub a piece of fabric between your hands for 15 seconds. Place this piece of fabric back into the plastic bag.*

2. *Please rub the cotton swab on the inside of your cheek, on both sides for 15 seconds. Place the swab back into the tube.*

3. *Please make a thumbprint on top of a glass square by placing your thumb onto the glass and pressing down firmly. Place the piece of glass back into the plastic bag.*

There are minimal risks to you for helping me with this project, as it is not designed to cause you any harm.

When I tell other people about this study, I will not use your name or other personal information.

Your [mom/dad/guardian] say that it is okay for you to help with my project. But if you don't want to help, you don't have to. No one will be upset if you say no. You can also stop helping me if you change your mind at any time.

Is there anything you don't understand about my project? Do you have any questions for me? You can call or email me if you have any questions later.

Would you like to participate in my study?

Note: Only a definitive "yes" answer from the child can be taken as oral assent to participate.

Child's Voluntary Response to Participate: _____ yes _____ no

Signature of Researcher: _____ **Date:** _____

(Optional) Signature of Child _____

Verbal Assent Form (child below 9 years old) – Touch DNA Sample
University of New Haven

Title: DNA methylation methods for donor age prediction in touch DNA samples
Investigator: Professor Timothy Palmbach J.D.
Co-Investigators: Dr. Claire Glynn, Emily Neverett, Kendra Jones

Parent/guardian must be present during the duration of sample collection for anyone under the age of 17 years old.

Name of Child:_____ **Date of Birth:**_____

Parental Permission on File: ____ yes ____no
(If **"no"** do not proceed with assent or research procedures)

Model Verbal Assent Script for Children (below 17 years old)
Instructions: This model provides suggested language to verbal assent for a child below the age of 17 years old. Child assent will be sought only after written parental informed consent for the child's participation is obtained.

Hi, my name is [researcher's name]. I am a [teacher/college student at the University of New Haven]. I am trying to learn if I can collect DNA from skin cells left behind on touched objects. I would like to ask for your help in the project, but before I do, I want to explain what will happen if you decide to help me. I will give you a sample collection kit which will contain the following items; a piece of fabric in a plastic bag, a cotton swab in a tube, and a piece of glass in a plastic bag.

To help with my project, please may I ask you to do three things:

1. *Please wash your hands with soap and water. Then, rub a piece of fabric between your hands for 15 seconds. Place this piece of fabric back into the plastic bag.*

2. *Please rub the cotton swab on the inside of your cheek, on both sides for 15 seconds. Place the swab back into the tube.*

3. *Please make a thumbprint on top of a glass square by placing your thumb onto the glass and pressing down firmly. Place the piece of glass back into the plastic bag.*

There are minimal risks to you for helping me with this project, as it is not designed to cause you any harm.

When I tell other people about this study, I will not use your name or other personal information.

Your [mom/dad/guardian] say that it is okay for you to help with my project. But if you don't want to help, you don't have to. No one will be upset if you say no. You can also stop helping me if you change your mind at any time.

Is there anything you don't understand about my project? Do you have any questions for me? You can call or email me if you have any questions later.

Would you like to help me with my project?

Note: Only a definitive "yes" answer from the child can be taken as oral assent to participate.

Child's Voluntary Response to Participate: _____ yes _____ no

Signature of Researcher: _____ **Date:** _____

(Optional) Signature of Child _____

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