2022

THE EFFECTS HUMIDITY & TEMPERATURE HAS ON DNA CONTAMINATION DURING STORAGE

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Dissertation

Presented in partial fulfillment of the requirements for the degree of

Doctorate of Philosophy in Anthropology

The University of Montana
Missoula, MT

September 9, 2022

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Ramey, Samantha, Ph.D., Fall 2022 *Anthropology*

The Effects Humidity & Temperature has on DNA contamination during storage

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Abstract

Studies have been conducted on DNA cross-contamination throughout the criminal investigation process in order to evaluation the possibility that DNA from one sample could potentially influence the outcome of another. However, no published studies have examined the potential for contamination during the storage of genetic evidence after samples have been taken from crime scenes or suspects. This study is a continuation of a preliminary project, which examined storage drying time in relation to cross-contamination. The current study tested temperature and humidity for the potential of cross-contamination during storage prior to extraction. Prior to storage, 50 µl of male saliva was aliquoted to buccal swabs and then dried for 20 minutes in a Dry-Fast swab dryer. Each variable tested consisted of five buccal swabs that were introduced to DNA and five swabs that remained unopened, in order to see if the male DNA could then be detected on the unopened swabs. Cross-contamination was not detected below 8 °C or 35% relative humidity. Any DNA that was detected was unable to produce a STR profile either as result of nonspecific amplification or extremely low levels of DNA, suggesting that if it did move, it was not enough DNA to alter results. However, further research is needed to determine if higher levels of humidity impact the movement of DNA.

ACKNOWLEDGMENTS

This research would not be possible without the support and guidance of several individuals. I want to thank my committee members Dr. Randal Skelton, Dr. Kelly Dixon, David Xing, and Joseph Pasternak, for their time, support, and feedback throughout this research. Thank you to my committee chair and advisor, Dr. Meradeth Snow, who was there through the ups and downs and many revisions that come with pursuing a Ph.D. Above all, I want to thank my family for supporting me to reach this point in my academic career.

I want to thank the organizations that provided the funding that supported this research. I am grateful for the funding provided by the Toelle-Bekken Family Memorial Fund and the ASUM Research & Creative Scholarship. Without their funding, this project would not have been possible.

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Chapter 1: Introduction

DNA is a vital and fragile genetic material used within many disciplines. With advances in genetic sequencing and analysis technology, DNA has been utilized more frequently in the court of law. Thus, the integrity of DNA is essential. The condition of DNA influences its stability, thereby influencing the preservation, which determines the success of identifying individuals, animals, plants, microorganisms, or food (Bonnet et al., 2009; Arenas et al., 2017). There is ample research discussing DNA contamination throughout different aspects of the collection process (Ladd et al., 1999; Pang and Cheung, 2007; Lapointe et al., 2015; Fonneløp et al., 2016; Pickrahn et al., 2017; Basset and Castella, 2018). The studies focus on the crime scene, collection, and extraction processes. However, there is a gap in the research concerning potential contamination during storage—specifically storage between collection and extraction. The research analyzing the storage process focuses on preserving DNA, but not considering potential contamination, or cross-contamination, from samples stored in close proximity to one another.

Because of the value of DNA, it is crucial to understand the optimal preservation methods. However, how genetic material is stored is dependent on the laboratory's protocols, which determine the packaging, storage conditions, and retention length of the evidence received at the forensic facilities (Ballou et al., 2013; Latta et al., 2015; Martin, 2016). If the appropriate storage conditions are not followed, then the integrity of genetic evidence becomes compromised, jeopardizing the information that can be obtained from the DNA. This risk is even more critical because of Hollywood's unrealistic portrayal of evidence, which leads jurors to expect genetic evidence to demonstrate immutable truth (Slabbert and Heathfield, 2018). Therefore, refining the storage conditions will benefit the preservation and integrity of genetic evidence, increasing the confidence of the genetic analysis.

A preliminary study was conducted to determine if cross-contamination can occur during storage. The study tested the storage length and drying time for two collection methods: swabs and Whatman cards by having one sample with DNA present stored in an evidence bag with a second that did not have DNA. The samples were stored at room temperature throughout the study. The longer Whatman cards were in storage, the more likely contamination occurred, and when analyzed with Fisher's Exact test the p-value was 0.00 when $\alpha = 0.05$ level. This indicates that storage length is statistically significant in the observation of contamination. However, the buccal swabs had a p-value of 0.054, which is not statistically significant at the $\alpha = 0.05$ level. The results from the study indicate that the longer Whatman cards are in storage, the more likely contamination is to occur (Ramey, 2019). The recommended storage conditions depend on storage length and evidence type, which some facilities cannot provide (Ballou et al., 2013; Latta et al., 2015; Martin, 2016). When stored, there is often no separation between evidence samples, with some facilities storing different cases in close proximity to each other (Cordray, 2010; Department of Public Safety - Texas, 2012; Ballou et al., 2013). Therefore, if the environment influences the DNA movement, then the potential for contamination increases when evidence is stored in close proximity.

The amount of time samples were left to dry after being exposed to wet DNA, or dry time, and DNA contamination were compared for the buccal swabs and the Whatman cards. The p-value for both the buccal swabs and the Whatman cards were greater than the significance level ($\alpha = 0.05$). This p-value shows that the longer samples are left to dry prior to storage does not decrease the potential of contamination.

In order to continue to investigate DNA contamination, the environmental conditions that were selected are those that protocols consider during the storage process: temperature and

humidity. Protocols provide four temperatures that evidence can be stored: frozen (at or below -10˚C), refrigeration (between 2˚C and 8 ˚C, less than 25% humidity), room temperature (ambient temperature), or temperature-controlled (between 15.5˚C and 24˚C, less than 60% humidity). The optimal storage temperature is dependent on the evidence type (Ballou et al., 2013). In contrast, humidity conditions are only referenced for refrigeration and temperature-controlled storage scenarios.

Temperature and humidity are the primary environmental factors that could potentially impact evidence throughout the storage process. When environmental factors are not considered, it puts DNA integrity at risk because certain environmental factors can cause damage to DNA (Alaeddini et al., 2010; Hall et al., 2014). Temperature has been found to be a factor in the movement of molecules (Widen et al., 2004; de Fátima Poças et al., 2011; Maia et al., 2016; Brandsch, 2017; Fang and Vitrac, 2017). Humidity has been shown to affect DNA's structure, beginning at 50% humidity (Westhof, 1988; Bonnet et al., 2009). Therefore, it is essential to research temperature and humidity concerning the DNA integrity during storage.

Chapters Outline: Chapters 2-4 provide background research discussing different areas for the project. Chapter 2 provides a general history of forensic genetics. The chapter also discusses the various DNA analyses that have been used over the years and how the information obtained from the analyses are utilized in the courtroom. Chapter 3 discusses the success rate and collection process of DNA from a crime scene. Chapter 4 discusses the preservation of DNA by analyzing environmental effects and storage methods. The chapter also discusses the movement of DNA and the migration modeling of molecules.

The subsequent chapters 5-8 will discuss the various aspects of the research. Chapter 5 discusses preliminary research and hypotheses, and outlines the storage process and DNA

analysis. Chapter 6 discusses the results from the various DNA analyses. Chapter 7 analyses the results by discussing the findings from the research. Finally, chapter 8 concludes the final remarks on the research and discusses how the study will impact the field in a forensic context.

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Chapter 2: Forensic Genetics

This chapter explores the history, DNA analysis methods, CODIS, and evidence in the courtroom of forensic genetics. The history of genetics extends over three centuries, but it was not until the second half of the 20th century that DNA would be used for forensics. Between the start of forensic genetics and now, various methods have been used to try and obtain a genetic profile for human identification. As the technologies advanced, DNA could be used in the courtroom as evidence, which would soon expand beyond just human identification.

Background

Most of the advances in forensic genetics have occurred in the past two decades, with the era of forensic DNA only beginning around 35 years ago. However, human identification has existed since the start of the 20th century through forensic serology. In 1900, Karl Landsteiner discovered the ABO blood groups (Li, 2018; Alessandrini et al., 2020; Erlich, 2020). This method of identification uses the antigens polymorphisms on the red blood cells. The antigens could aid in identification based on varying frequencies of the four blood types within a population; the disadvantage is the power of discrimination because ABO blood groups can only exclude individuals from identification, not confirm them (Alessandrini et al., 2020).

A decade after Landsteiner's discovery, Edmond Locard established Locard's Principle of Exchange in 1910. The principle states, "every contact leaves a trace" (Rutty and EAM, 2005; Byard et al., 2016; Li, 2018; Allwood et al., 2020). Modern forensic genetics relies on the trace DNA found at crime scenes to aid in the investigation. Thus, Locard's principle helped establish this idea of traceable identification (Byard et al., 2016; Li, 2018; Mistek et al., 2019). In 1917, Thomas Hunt Morgan published his theory of the gene (Morgan, 1917; Li, 2018). His theory

further examined the Mendelian law of heredity and discovered that genes are located on the chromosomes and are the basic unit of heredity (Morgan, 1917), establishing the foundation of forensic genetics, and the field of genetics as a whole (Li, 2018). However, serology would be the source of human identification until the mid-1980s. In 1927, Landsteiner and Levine discovered two antigens P (Globoside) and MNSs blood system. By the 1930s, red blood cells were still used for human identification. During this time, Levine and Stetson discovered the LW blood system, followed shortly by the discovery of the Rh factor by Alexander Weiner and Landsteiner. In total, sixteen red blood cell antigens would eventually be used in identification, providing a modest power of discrimination. By the 1960s, around 60 serological markers were used for identification. These markers now included the white blood cell antigen histocompatibility known as HLA (Pourazar, 2007; Alessandrini et al., 2020).

In 1953, the structure of DNA was discovered to be double-helical (Li, 2018). This discovery was a significant advancement toward the use of DNA for identification and forensic genetics (Reich et al., 2002; Li, 2018). In 1984, Dr. Alec Jeffreys discovered 'DNA fingerprinting,' now known as DNA profiling. Jeffreys found DNA heritable patterns that resemble a barcode, which the comparison was seen after completing southern blot analysis. The targeted segments of the DNA separate by the agarose electrophoresis based on the size of the amplified DNA, in which smaller DNA will travel faster through the gel, resulting in bands (Roewer, 2013; Zahra et al., 2018; Carracedo and Prieto, 2019; Alessandrini et al., 2020; Bright et al., 2020). This discovery began the era of forensic DNA, and the following decade's research focused on exploring DNA profiling (Roewer, 2013). With DNA profiling, genetic evidence could now provide the ability to discriminate between individuals based on a likelihood ratio ([LR]; Carracedo and Prieto, 2019; Bright et al., 2020). The ratio can communicate the

significance of the DNA by measuring the probability of the genetic evidence belonging to a suspect (Carracedo and Prieto, 2019). This was most important to understand when the use of DNA was first introduced to the courts because DNA profiling was a new concept. The first case to use DNA profiling involved an immigration issue where a boy was at risk of deportation, but with DNA evidence, he was saved. The boy was thought to be either a nephew or unrelated to a woman living in the United Kingdom. The conventional genetic markers for the time (ABO blood group, Rh, HLA, etc.) indicated the two were related. However, the analysis could not confirm if the boy was her son, and a DNA profile was able to prove their true relationship (Jeffreys et al., 1985). Jeffreys stated, "If our first case had been forensic I believe it would have been challenged and the process may well have been damaged in the courts" (Roewer, 2013). This public acceptance of DNA profiling paved the way for the data to be used in forensic cases.

The DNA now being used for human identification instead of forensic serology created a need for a place to access it. In 1995, England created the first DNA profile bank. This was soon followed by Northern Ireland, Scotland, and New Zealand in 1996 (Carracedo and Prieto, 2019). The databases are beneficial to law enforcement by providing an individual's unique marker identifiers. In 2004, Kirk Bloodsworth became the first death row inmate to be exonerated with DNA (Junkin, 2005). However, the use of DNA databases raises concerns from the public, despite their expectation of using genetic evidence within the courtroom. The general public has three main concerns regarding the databases: a lack of DNA data transparency, lack of international standardization of DNA analysis, and potential ethical oversight. Ethical concerns increase when considering forensic DNA phenotyping (FDP) because of the potential stigmatization of specific populations (Machado and Silva, 2019). These concerns still exist

today but could potentially be resolved through transparent dialogue with the general public about the DNA extraction and analyzing process.

Until 2005, forensic genetics was still focusing on the standardization of the field. However, the 2000s began the rapid advancements in forensic genetics that occurred between 2005 through 2015, focusing on new technologies and applications (Roewer, 2013; Butler, 2015). Databases were expanded, and by the end of this period the United States National DNA Index System (NDIS) grew by 12 million genetic profiles. New STR kits were implemented in Europe and the United States. Instruments pursued rapid DNA profiling (Butler, 2015).

The expansion of these new technologies allowed forensic genetics to become sophisticated in less explored areas beginning in 2015 (Butler, 2015). Nonhuman genetic elements like food, animals, microorganisms, and plants, which have overlapping applications in forensics, were applied in a forensic context. Plants, microorganisms, and animals can all be silent witnesses of crimes. The silent witness is the genetic evidence left behind that is not a person's DNA, such as pet hair, soil DNA, and grass. Food, microorganisms, and plants are also applicable for bioterrorism (Arenas et al., 2017). Law enforcement now uses these newer areas to identify wildlife, hunting, and food authentication (Amorim et al., 2020). Despite nonhuman forensic genetics, there are still limitations due to minimal species having been identified and established for comparison (Arenas et al., 2017).

Advancements in technologies have also allowed investigators to generate a phenotype report based on an individual's DNA (Kayser, 2015; Hopman and M'charek, 2020). This began with the probability of an individual's eye and hair color (Kayser, 2015). There are analysis services that provide a phenotype report consisting of sex, ancestry, skin color, eye color, hair

color, freckle percentage, and facial reconstruction (Hopman and M'charek, 2020). Therefore, as the methods and technologies advance, more information is obtained from genetics.

DNA Analysis

Retired

The recent advancements in the technology for DNA analysis and extractions have caused RFLP, DQ alpha, and AmpFLP to become retired technological methods. These retired methods were used in forensics to generate genetic profiles but became obsolete in forensic genetics for various reasons.

RFLP

Restriction fragment length polymorphism (RFLP) was the first method to analyze a pattern in the variation of tandem repeats, thus creating a genetic profile beginning in 1980 (Vitoševic et al., 2019; Dash et al., 2020a). Today, this method has been replaced with polymerase chain reaction (PCR) based technologies. The process follows four steps: first, the DNA is cut into fragments using restriction endonuclease. Next, the fragments were separated with gel electrophoresis. Then the fragments are transferred to a nitrocellulose membrane to conduct a southern blot and finally analyzed using radioactive probes (Vitoševic et al., 2019). The DNA required for a successful RFLP analysis consisted of large quantities of intact DNA, making the method not optimal for forensics (Roewer, 2013; Børsting and Morling, 2015; Vitoševic et al., 2019; Erlich, 2020).

DQ alpha

In 1991, the DQ-alpha test was developed to examine the poly-allelic locus of the HLA-DQA1 gene (Tilstone et al., 2006). Because this method used PCR, it did not require the same

quantity of DNA as RFLP, which seemed promising for forensic cases. However, the process was labor-intensive, detected sequence variation, and the discriminatory power was not optimal for forensic analysis (Saiki et al., 1986; Tilstone et al., 2006; Erlich, 2020). The analysis of this method consists of eleven probe dot-blot assays (alleles: 1, 2, 3, 4, C, 1.1 [1.2, 1.3, 4], 1.3, all but 1.3, 4.1, [4.2, 4.3]), in which the intensity of the color the dot turns indicates the amount of amplified DNA bound to the specific probe. The results were then compared to other samples; however, the intensity of the dot colors could potentially be interpreted differently (Erlich, 2020).

AmpFLP

In the 1990s, Amplified fragment length polymorphism (AmpFLP) was developed using PCR to generate a DNA fingerprint with dominant markers. The method was fast, easy, replicable, and relatively cheap for the time. Compared to RFLP, the quantity of information was higher. Preparing the DNA to generate AmpFLP markers consisted of template preparation, restriction and ligation, and selective amplification. The AmpFLP markers allowed multiple polymorphic bands to be analyzed in one gel lane simultaneously. Therefore, the bands of different samples would be compared to each other (Blears et al., 1998; Mueller and Wolfenbarger, 1999).

Current Methods

STR

Short tandem repeat (STR) is currently the primary method used in forensic genetics (McCord et al., 2019). STRs are found throughout the entire genome, containing 2 – 7 base pairs that repeat in tandem for a various number of times (Panneerchelvam and Norazmi, 2003; Vitoševic et al., 2019; Dash et al., 2020a). STR analysis methodology expands on the retired RFLP method because STR is more sensitive, and AmpFLP is prone to allelic drop-out (Roewer,

2013). An advantage to STRs is that they can be amplified simultaneously in single multiplex amplification and detect a mixed sample (Butler et al., 2007). The process of generating an STR profile follows four steps: DNA isolation, amplification, electrophoresis, and data analysis. The profiles are then used to compare to the profiles of various samples (Turnbough et al., 2013; McCord et al., 2019). STRs have a high mutation rate of approximately 10^{-3}, which is a limitation because it makes STRs less stable; however, it is more discriminatory than other techniques (Butler et al., 2007; Roewer, 2013; Vitoševic et al., 2019). In comparison, the average mutation rate per nucleotide site ranges between 1.6×10^{-7} to 2.3×10^{-9} (Nachman and Crowell, 2000). In forensics, the markers used are located in the non-coding region of the genome and the first markers used were TH01, vWA, FES/FPS, and F13A1 because of their simplistic repeat sequences (Wyner et al., 2020).

In 1992, the first Y-STRs were discovered. Now, a few forensic kits include Y-STRs (Kayser, 2017). These types of STRs are only found in biological males, which is beneficial when dealing with paternal relationships or mixed male/female samples (Diegoli, 2015; Vitoševic et al., 2019). The addition of Y-STRs to the normal autosomal STRs has enhanced DNA analysis. However, because of the low mutation rate, approximately $2 – 4 \times 10^{-3}$, and the lack of recombination, Y-STRs cannot discriminate between related men. In comparison, the average X STR mutation rate is 1.35×10^{-3} (Diegoli, 2015; Kayser, 2017), resulting in more mutational differences and, therefore, higher discriminatory power between individuals.

There are potential problems with STR readings: allele drop-out, drop-in, or stutter, which can interfere with the interpretation of the genetic profile. If these problems occur, it can be challenging to determine if the sample is a mixture of DNA, indicating some contamination (McCord et al., 2019). Allele drop-out is when there is no allele observed at the locus or the loss

of one of the alleles leading to the appearance of homozygosity. This is common when there is low template DNA. It is difficult to observe, often leading to the requirement to run a sample multiple times to ensure homozygosity or that all alleles are accounted for. Allele drop-in is an additional peak typically smaller than prominent peaks at a given locus, originating from extraneous DNA from another DNA sample. With an allele stutter, the peak will not appear in its intended position, and the new position will depend on the peak size (Balding and Buckleton, 2009; Buckleton, 2009; Taylor et al., 2014). It may be possible to determine that the sample is a mixture based on various alleles if there are no overlapping alleles of the multiple DNA contributors (Butler et al., 2007). However, if the contributors share an allele, this is known as masking (Taylor et al., 2014).

Another potential problem is the two types of tri-allelic patterns at STR loci. In the first type, the three peaks are uneven, but the sum of the smaller peaks equals the height of the most prominent peak. The second type is divided into two patterns 1:1:1 or 2:1. In the first pattern (1:1:1), the three peaks are equal in height. While in the second pattern (2:1), there are two peaks, with one being double the height because it consists of two identical alleles (Yang et al., 2020).

mtDNA

Mitochondrial DNA (mtDNA) is maternally inherited and analysis of it is a current method in forensic genetics. In forensics, the process of generating a mtDNA analysis typically follows Sanger sequencing and the most important step is the pre-extraction sample prep (Holland et al., 2013). MtDNA is beneficial if the samples are highly degraded because of the many copies per cell compared to the two copies of nuclear DNA (Vitoševic et al., 2019). However, it is less discriminating than STRs because of the lack of recombination since there is

only a single marker maternally inherited and therefore used only when nuclear DNA is unavailable (Holland et al., 2013; McCord et al., 2019; Vitoševic et al., 2019).

MPS

Massively parallel sequencing (MPS) is known by several names in forensics and used interchangeably, such as next-generation sequencings and high throughput sequencing (Erlich et al., 2020). Between 2005 to 2007, several systems using MPS were introduced (Bruijns et al., 2018; Arora, 2020). The technology has a higher throughput compared to Sanger sequencing, which is not frequently used with forensic human samples, has accurate sequencing capabilities potential, rapid processing, and is low-cost. The different MPS systems follow the key steps: library preparation, template DNA amplification and distribution, sequencing and imaging, base calling, quality control, and data analysis (Berglund et al., 2011; Zascage et al., 2013; Murphy, 2018; Kumar et al., 2019; Erlich et al., 2020). There are advantages to using MPS over other methods. Unlike STRs, MPS examines each nitrogenous base of the nucleotide of a region (Murphy, 2018). MPS systems sequence reactions simultaneously and initiated with one DNA molecule; because of this, MPS can be applied to analyze various genetic markers (McCord et al., 2019; Novroski et al., 2019; Erlich et al., 2020). Despite these advantages, MPS is rarely used in forensics and primarily limited to research studies. But the consensus among academics is that MPS has the potential of impacting forensics (Bruijns et al., 2018; Murphy, 2018; Erlich et al., 2020). In 2019, the NDIS Board of the FBI approved the uploading of MPS kits to DNA databases. However, the NDIS can only store, upload, and search the required CODIS Core Loci and NDIS accepted loci. This was seen in a European laboratories survey where four challenges emerged when implementing MPS instruments in their labs used to analyze identity, ancestry, or autosomal-STR markers: no reporting standards, not compatible with existing national DNA

databases, insufficient population data for statistical calculations, and minimal legislative framework (Alonso et al., 2017; Butler and Willis, 2020).

Rapid DNA

As the name suggests, rapid DNA is capable of producing a DNA profile within two hours. The sequencing is limited to generating an STR profile at a set number of loci because of its speed. For instance, DNAscan 6C can analyze 27 loci under two hours (Dash et al., 2020a; Erlich et al., 2020). These are typically machines, the size of a desktop printer, that are simple to use and require no special training to operate (Murphy, 2018). The process is quick and straightforward, following six steps that are completed through this one machine: signal processing, fragment identification, comparison with internal lane standard, comparison with allelic ladder, locus and sample specific analysis, and generated profile (Dash et al., 2020a; Chong et al., 2021). However, the sample must be of high-quality DNA and from a swab (Erlich et al., 2020). The analysis is intended for samples from a known individual due to the fast nature of the sequencing. Therefore, rapid DNA is used to search against a DNA database of an arrested suspect. The Rapid DNA Act of 2017 outlined guidelines for this technology, which is an amendment to the DNA Identification Act of 1994. Rapid DNA analysis is no longer required to be conducted in a qualified laboratory if the guidelines of when uploading or searching DNA databases with rapid DNA can occur are followed. Samples are now required to be reference samples and not a forensic sample (Anon, 2017; Murphy, 2018; Butler and Willis, 2020; Erlich et al., 2020)

CODIS

The United States has influenced the standardized genetic markers used in the identification of a genetic profile. As discussed earlier, STRs are short tandem repeats used to establish the standardized markers and first used in 1991 (Zhang et al., 2020). The DNA Identification Act of 1994 allowed the United States' FBI to establish a national database to store DNA profiles known as CODIS, the Combined DNA index system (Butler and Li, 2014; Karantzali et al., 2019). In 1998, the first 13 standardized loci were introduced: D8S1179, D21S11, D5S818, CSF1PO, D3S1358, TH01, D13S317, D16S539, TPOX, D18S51, vWA, D7S820, and FGA. Then in 2001, the European standard set was established with 7 of the genetic loci from the CODIS set: D8S1179, D21S11, D3S1358, TH01, D18S51, vWA, FGA. In the last decade, there were discussions of expanding the current CODIS standard set to reduce the number of adventitious matches. Validation studies were conducted on three PCR amplification kits: Life Technologies' GlobalFiler, Life Technologies, GlobalFiler Express, and Promega Powerplex. Based on the validation data, it was concluded that the expansion should retain the original 13 loci and added seven additional loci: D1S1656, D2S441, D2S1338, D10S1248, D12S391, D19S433 and D22S1045 (Hares, 2015; Karantzali et al., 2019; Butler and Willis, 2020). The FBI required the additional STR loci to be implemented into genetic labs by January 1, 2017 (Hares, 2015; Moretti et al., 2016).

SE33 and Amelogenin were two of the loci considered; however, these loci did not qualify for the CODIS expansion. However, both markers are beneficial for familial DNA searches (paternity testing, missing people, etc.). SE33 is a highly polymorphic locus, located on chromosome 6 (6q14). Studies have indicated that SE33 can exclude false matches and increase the true positive rate to false positive rate ratio (Butler et al., 2009; Bhinder et al., 2018;

Karantzali et al., 2019). Unlike SE33, Amelogenin has incorrectly determined the individual's biological sex in many cases, despite it being required for genetic profiles of relatives of missing persons or unidentified human remains along with the standardized CODIS loci (Butler and Li, 2014). Amelogenin has two homologous genes AMELX, located on the X chromosome, and AMELY, located on the Y chromosome, which failure to amplify AMELY suggests the absence of the Y chromosome (Steinlechner et al., 2002; Davis et al., 2012; Butler and Li, 2014; Ge et al., 2014). However, CODIS has not decided to include other known advantageous alternatives to Amelogenin.

What role does CODIS have in forensic genetics? CODIS is the national DNA database in the United States comprised of three levels: local DNA index system (LDIS), state DNA index system (SDIS), and national DNA index (NDIS; Butler and Li, 2014). Each system allows DNA profiles to be exchanged and compared at the system's designated level (Budowle et al., 1998). Most countries have their own form of a forensic DNA database similar to CODIS. These databases typically contain two types of profiles: reference profiles, and forensic profiles (Ge et al., 2014; Arora, 2020). Databases are used to run reference DNA samples against the unknown forensic profiles for possible profile matches, which does not always result in a match. For example, it took five years before the Macedonia National DNA database resulted in a possible match in one case report. The suspect was caught because of the account of an unrelated crime five years after the initial case. While another case using the same database, a possible match was already in the system when the unknown sample was run against the database (Jakovski et al., 2017). Therefore, DNA databases need to be run regularly as new profiles are being continuously added in order to check for unsuspected matches.

Courtroom and new evidence

The first use of DNA was not a forensic case, as mentioned earlier. The outcome of that case was monumental in paving the way for the use of DNA in the courtroom in a forensic context. The first forensic application was in 1987 in England (Visser and Hampikian, 2012; Roewer, 2013). In 1996, the United States court system accepted the use of human mtDNA (Lyons et al., 2014). However, despite the advancements in forensic genetics, people have still been wrongly convicted based on genetic evidence. For example, in Australia, a boy was convicted with DNA that had unknowingly been contaminated during the collection process (Weathered et al., 2020). Genetic evidence can be useless if the database collection is limited, there is a backlog of samples, profiles are not uploaded to CODIS, or data is misunderstood (Visser and Hampikian, 2012). Yet, in the court system DNA is still the gold standard of evidence. Therefore, it is essential to understand the public's view of DNA (Visser and Hampikian, 2012; Weathered et al., 2020). The "CSI Effect" is an increasing phenomenon that occurs because jurors have an unrealistic expectation of evidence due to television and expect DNA evidence to be entirely foolproof (Slabbert and Heathfield, 2018).

Today, DNA can be used in a forensic context beyond human applications. As briefly discussed earlier, nonhuman elements are being applied to a forensic context. There are differences seen in these uses compared to human DNA. For instance, different questions are asked in wildlife DNA forensics, with some common questions: what is the species? is it wild or captive-bred? or where is it from? These questions are then used to protect wildlife with various laws and treaties (Moore and Frazier, 2019). Forensic genetics can then be applied in cases such as protected species, food fraud, and poaching (Arenas et al., 2017; Amorim, 2019). In 2016, an investigation of a bone necklace of expected whalebone (protected as an endangered species) led

to the conviction of a couple on a variety of accounts, including Lacey Acts violations, which prohibits the trade of illegally acquired wildlife (Moore and Frazier, 2019). In 2009, the mitochondria DNA of cat hair was first used in the murder trial of the State of Missouri versus Henry L. Polk Jr., linking the suspect to the victim. At the time of the case, this specific cat had a rare mitotype to allow the evidence to be admissible. However, now the dataset of USA cats is sufficient for more general forensic applications (Lyons et al., 2014). Because of nonhuman DNA, suspects can be linked to the crime. For example, pollen and fungal spores have also been used to link a suspect to the scene from the palynological material on their shoes, which contradicted the suspect's statements (Allwood et al., 2020).

Probabilities and Likelihoods

When presenting the genetic evidence to a courtroom, its value is indicated with a likelihood ratio. As mentioned earlier, the likelihood ratio communicates the probability of genetic evidence belonging to a suspect (Caliebe et al., 2017; Carracedo and Prieto, 2019). However, the ratio is not a probability according to the definition of probability theory, since the likelihood ratio is not additive; instead, it measures the 'rational belief' of the two likelihood hypotheses (Caliebe et al., 2017).

A recent analysis of probabilities indicated concern with the use of the likelihood ratio in the courtroom. The current probability method is divided into five groups, beginning with 1 to 10 and ending with 10,000 or greater. This ratio range indicates the evidence provides limited support through very strong support that the genetic material came from the suspect (Roberston et al., 2016; Weathered et al., 2020). Based on this presentation of statistical evidence, two concerns were observed. First, the likelihood ratio presentation to jury members was significantly more challenging to understand than random match probability (RMPs). In the

study, the jury correctly interpreted 42% of the DNA evidence presented as likelihood ratios. In comparison, 83% of the DNA evidence presented as RMPs was correctly interpreted (Weathered et al., 2020). Second, the language and definition of a "partial profile" and "complete profile." The completeness of the profile does not indicate the number of loci used to create the profile. Therefore, the likelihood ratio can theoretically be the same for both a partial and complete profile. For example, a loci kit can use either 9 or 21 loci markers. If a profile were comprised of 9 loci, then depending on the kit, the 9 loci kit would generate a complete profile compared to a partial profile for the 21 loci kit. Without this distinction, part of the evidence context is obscured from the jury and can confuse them, potentially leading to problematic verdicts (Weathered et al., 2020).

With the advances in analyzing DNA, additional probabilities have been introduced to the criminal justice process. Forensic DNA phenotyping is a relatively new process that predicts externally visible characteristics (Caliebe et al., 2017; Aggarwal, 2020). The method analyzes single nucleotide polymorphisms to determine the phenotypes using posterior odds, which contradicts the standard forensic reporting of only likelihood ratios in court. This probability for FDP is used because the odds are independent of the population. Posterior odds are not currently reported and used primarily by law enforcement, with few exceptions depending on the evidence (Caliebe et al., 2017).

Unlike DNA profiles, FDP is not meant to be used as evidence because there are limitations and ethical concerns about the FDA beyond its visible characteristic predictions (Caliebe et al., 2017). The phenotypic report cannot estimate potential environmental factors that can affect the genes, which can change the visible characteristics. The accuracy of the reports decreases with profiles of mixed ancestry (Aggarwal, 2020). These limitations support the

expressed European concerns about the potential of FDP reports being misunderstood (Samuel and Prainsack, 2019). Another concern is the privacy of the individual (Toom et al., 2016; Slabbert and Heathfield, 2018; Samuel and Prainsack, 2019; Aggarwal, 2020). The regulation of FDP is varying; for instance, the Netherlands allows the use of externally visible characteristics but is limited to traits visible from birth that can contribute to the investigation, while South Africa restricts the use of externally visible characteristics (Slabbert and Heathfield, 2018; Wienroth, 2018). These points are essential to consider because of the "CSI Effect" phenomenon since FDP is sometimes viewed as a "biological witness" (Kayser, 2015; Slabbert and Heathfield, 2018; Machado and Granja, 2020).

Conclusion

Forensic genetics has a long history spanning several decades, with the most recent advances being within the last decade. Understanding its history allows new questions to be explored, and being able to understand the basics of each method allows for the integrity of the DNA to be maintained throughout the forensic process. Even with recent advances in technologies and methodologies, new concerns will arise from the general public. Research must continue to focus on these current advances to address potential problems and allow scientist to return to concepts not once considered.

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Chapter 3: Crime Scene Collection of DNA

Over the past decade, the process of collecting genetic evidence has made significant advances. Today, more DNA can be collected at lower quantities, and a genetic profile can still be reliably ascertained. In theory, this allows more crimes to be solved with the use of DNA. However, the benefit of genetic evidence in forensics is more complicated than merely collecting the DNA and must follow requirements to be effective: i) the collection technique (e.g., swab, tape), ii) the collection surface where the DNA is located (e.g., glass, wood), iii) the source of the DNA (e.g., blood, saliva, touch DNA), iv) and the sampling strategy to achieve a high success rate of a genetic profile.

These collection requirements are interrelated. While each element needs to be considered, the sampling strategy must be determined on the elements together. For instance, a saliva sample from a glass surface collected using a swab will result in different success rates based on degradation (Lee and Ladd, 2001; Hogan et al., 2018). The DNA source can also be the same, but the surface type results in various DNA amounts (Lowe et al., 2002; Raymond et al., 2009; Goray et al., 2010; Daly et al., 2012; Poetsch et al., 2013, 2018). Based on this knowledge, consistent success rates can be achieved by selecting the proper sampling strategy.

Success Rate

The success rate is the ability to generate a genetic profile from the evidence collected at a crime scene, which is dependent on the collection strategy (Zuidberg et al., 2019). This is accomplished by considering the four elements: DNA source, collection methods and materials, collection surface, and contact location (Baechler, 2016; Hess and Haas, 2017; Dziak et al., 2018; Zuidberg et al., 2019). Without considering these elements, the success rate varies. For

instance, a study was done comparing three collection techniques, foam swab, blood FTA card, and the saliva Oragene DNA (OG-500) kit. Despite the saliva collection method recovering a significantly greater DNA yield, the technique also had the highest contamination rate (Prasad and Vardhanan, 2018). Of the main four elements, the most critical success rate is the collection method and location (Mapes et al., 2016; Hess and Haas, 2017; Zuidberg et al., 2019).

There are natural factors that cause concern for the recovery of DNA from a crime scene. The potential of these factors affecting the success rate is present for all genetic evidence. These factors are degradation, concentration, and purity of the DNA (Lee and Ladd, 2001; Cătălin et al., 2011; Mapes et al., 2016). First, as observed with the collection from various substrates, DNA degradation has a significant impact on the success rate and can potentially influence the collection process (Lee and Ladd, 2001; Cătălin et al., 2011; Aloraer et al., 2015). Second, the concentration of DNA can help determine the expected success rate. For example, touch DNA has a low concentration of DNA; therefore, it is expected to have a low success rate (Mapes et al., 2016). However, this expectation can only be accepted if the collection is free of human errors. The third concern is purity during the collection process. The purity of the DNA is similar to the concern of contamination. However, an impure sample could result from a variety of sources such as dirt, dyes, or bacterial DNA (Lee and Ladd, 2001; Mapes et al., 2016). By considering these natural concerns during every sampling strategy, then the potential for a better success rate increases.

Law enforcement agencies

Investigators or police officers do the collection of evidence. Therefore, they must be knowledgeable about the various sampling strategies because forensic DNA analysis can only work with what is left at a crime scene. Many investigators still depend on experience from prior

casework to collect DNA (Baechler, 2016). The experience of the investigator can then, in turn, influence the DNA success rate (Wood et al., 2017). Training should be completed before an individual can collect evidence with annual refreshers to ensure the highest success rate (Storm et al., 2009; Hauhart and Menius, 2014). Insufficient training and knowledge for the collection of genetic evidence will increase the potential for contamination.

The protocols for various laboratories do not provide the necessary information for a collection sampling strategy. If evidence collection practices are included in the protocol, then the information is limited to swab samples (Cordray, 2010; Cătălin et al., 2011; Department of Public Safety - Texas, 2012; Ballou et al., 2013). Nevertheless, with more funding, training, and research, understanding the benefits of genetic evidence will advance.

The current collection strategy used by law enforcement and crime scene investigators is singular. The strategy focuses solely on the collection method primarily the double swab technique consisting of a wet swab followed by a dry swab (Hedman et al., 2020). It would be advantageous to train investigators and law enforcement on the proper collection strategy to optimize DNA collection (van Oorschot, 2012; Adamowicz et al., 2014; Hauhart and Menius, 2014; Lapointe et al., 2015). This training should focus on the collection process, transport, storage, and contamination (Cătălin et al., 2011; van Oorschot, 2012). Together these will benefit the DNA analysis success rate.

Contamination

There is extensive research understanding the potential contamination during the collection process. Despite this, the contamination rate is increasing, which began after next-generation multiplex (NGM) began being implemented. NGM more likely enhanced the accuracy of the contamination rate because of its sensitive analysis of DNA, which has increased

the indication of contamination (Kloosterman et al., 2014; Fonneløp et al., 2016; Pickrahn et al., 2017; Basset and Castella, 2018). A high percentage of the contamination originates during the collection process from individuals handling evidence or swabbing (Lapointe et al., 2015). These contaminations could also result from an insufficient understanding of the benefits of genetic evidence and insufficient funding (Storm et al., 2009; Blozis, 2014; Hauhart and Menius, 2014).

DNA Source

When collecting DNA, it can originate from different sources, such as touch DNA, saliva, or blood, which can influence the collection method. Before 2001, the analysis was limited to evidence with nucleated cells (Lee and Ladd, 2001). Now analysis includes other DNA sources. It is essential to consider the source of genetic evidence before collecting it because this will influence the quality, quantity, and preservation of the DNA (Cătălin et al., 2011; Hauhart and Menius, 2014). Collection methods and materials are not always interchangeable. Therefore, the DNA yield variability is potentially caused by the collection method rather than low DNA levels (Barash et al., 2010; Kirgiz and Calloway, 2017). What must be known about the genetic evidence, before choosing the collection method? It depends on the source of the DNA. When collecting touch DNA, more information is needed to understand its biological source (skin, sweat, etc.) in comparison to blood or saliva. Therefore, the collection methods are more likely to be different between these sources of DNA.

Knowing the DNA source can help identify which genetic evidence might have the highest success rate. Thus, a collection order can be established to determine the order of importance based on the probability of DNA's presence, and its sensitivity when collecting genetic evidence. Therefore, trace DNA (handled objects) should be collected first, followed by

facial bodily fluid (e.g., glassware, utensils), then blood, and lastly semen and tissue (Wickenheiser, 2002). Touch DNA yields low levels of loci, and one study found that 50% of samples have had 9 loci or less, thus, the reason it needs to be collected first (Baechler, 2016). Despite the amount of DNA amplified from a pure sample, the range can differ from samples collected from a crime scene (Cătălin et al., 2011; Tredoux et al., 2015; Baechler, 2016). Tredoux et al. (2015) found that buccal samples significantly yield more DNA than blood samples. The amount of DNA from contact with the surface will vary between individuals; however, this typically refers to the transfer of skin cells.

The potential DNA source from touch DNA consisted of five sources: cell-free DNA, anucleate corneocytes, fragmentary cells, nucleated cells, and epithelial cells (Burrill et al., 2019). For biological fluid (i.e., blood, semen), the DNA source is known once it is identified. However, touch DNA may consist of multiple DNA sources, and knowing the DNA source allows for better insight into the collection (Wickenheiser, 2002; Quinones and Daniel, 2012; Zoppis et al., 2014; Ostojic and Wurmbach, 2017). Cell-free DNA was discovered in 1948 in the plasma and now known to be found in biological media, such as blood and urine (Mandel and Metais, 1948; Vandewoestyne et al., 2013; Burrill et al., 2019). Thus, it is crucial to know where the DNA originates because the biological evidence can become cell-free from degradation and therefore increase fragmentation after transferring (Burrill et al., 2019). Many studies found that cell-free DNA has the potential to enhance forensic casework since it can increase the DNA yield (Kita et al., 2008; Linacre et al., 2010; Quinones and Daniel, 2012; Vandewoestyne et al., 2013). Anucleate corneocytes are the outer layer of the epidermis cells that have keratinized that are thought to be nuclei free. Therefore, they are not considered significant despite being the primary cell source in the epidermis (Quinones and Daniel, 2012; Burrill et al., 2019).

Fragmentary cells are single-stranded DNA that includes stripped nuclei (Kita et al., 2008; Zoppis et al., 2014; Burrill et al., 2019). However, there is a contradiction between the source of the fragments. Zoppis et al. (2014) found the cells forming in the sebaceous gland. This supports the importance of sebaceous as a vector. In contrast, Kita et al. (2008) found the fragments localized in the cornified layer located within the epidermis layer (Burrill et al., 2019).

DNA Transfer

The transfer of DNA to an object through the loss of skin cells is complicated. Four factors impact the amount of DNA transfer: i) contact force, ii) prior activities, iii) the object, iv) the individual (Lowe et al., 2002; Pang and Cheung, 2007; Daly et al., 2012). Many studies sought to understand why individuals shed epithelial cells differently, but data is still inconclusive (van Oorschot and Jones, 1997; Ladd et al., 1999; Lowe et al., 2002; Quinque et al., 2006; Pang and Cheung, 2007; Raymond et al., 2009; Goray et al., 2010; Daly et al., 2012; Kamphausen et al., 2012; Poetsch et al., 2013). Kamphausen et al. (2012) concluded that the health of the individual's skin might impact the rate of shedding skin cells. Poetics et al. (2013) found by comparing children (1-10 years old) and elders (61 and older) that there was a correlation between the quality and quantity of DNA shed by the individual. A few studies suggest that the quantity of DNA shed depends on the contact pressure, tendency to shed, and prior activities (Ladd et al., 1999; Lowe et al., 2002; Pang and Cheung, 2007). These cells are shed daily and would be ubiquitous at a crime scene due to their constant loss. Other DNA sources will be present at a crime scene based on the situation. Therefore, touch DNA is the most commonly researched type of biological evidence, yet it remains one of the most difficult to use.

What causes the cells to transfer? On average, around 400,000 skin cells are shed every day by an individual (Hess and Haas, 2017). This shedding process begins with cells moving

from the basal layer through the epidermal layer as new cells form. These older cells eventually will form into keratinocytes and thought to leave the traces of DNA. These results indicate the source of DNA transfer for a primary transfer will originate from the keratinocytes. This transfer will occur from direct contact with an object (Zoppis et al., 2014; Cale et al., 2016; Helmus et al., 2016; Ostojic and Wurmbach, 2017; Pickrahn et al., 2017). However, Zoppis et al. (2014) found this untrue when analyzing secondary DNA transfer. A secondary transfer occurs from indirect contact with an object (Cale et al., 2016; Helmus et al., 2016; Pickrahn et al., 2017). In this instance, the DNA originates from the sebaceous glands that produce a substance comprised of cellular debris (Zoppis et al., 2014; Vickar et al., 2018).

Touch DNA is typically referring to the cells transferred to an object. It is the DNA obtained at low trace levels from cellular and cell-free DNA (Hanson and Ballantyne, 2013; Vickar et al., 2018; Sessa et al., 2019). Unlike other sources of DNA (blood, saliva, etc.), touch DNA presents four difficulties in collection: i) quantity deposited, ii) quality deposited, iii) visibility, iv) and lack of a presumptive test for all surfaces (Verdon et al., 2014; Kirgiz and Calloway, 2017; Hefetz et al., 2019). The quantity of deposited DNA is also influenced by the individual, the substrate, and contact pressure. A study found that the quality of DNA deposited from latent fingerprints improves with increased contact pressure between 0 kg to 2 kg (Hefetz et al., 2019). There are three approaches when targeting touch DNA since there is a lack of presumptive tests to indicate if DNA is present. The first approach relies on prior cases, looking at the success rate of various sample locations for similar items. If prior data is limited, then the approach is limited. The second approach relies on visualization, which focuses primarily on detection methods. The final approach relies on information on the specific case. This approach

is limited to the information available on the case (Zuidberg et al., 2019). Due to these difficulties, the DNA must be collected methodically to allow the best success rate.

Blood, saliva, and other DNA sources

Unlike touch DNA, other biological materials have various DNA sources. For instance, saliva contains the same epithelial cells as touch DNA, blood contains leukocytes, and bone contains osteocytes (Quinque et al., 2006; Dash et al., 2020b). Nevertheless, epithelial cells are the primary source of DNA from a crime scene. The cells are found in sweat, vaginal fluid, and saliva (Dash et al., 2020b). The state of these biological materials, other than touch DNA, can also vary during the collection process. The biological sample will either be a liquid, dried stain, soft tissue, or hard tissues (i.e., bone, hair) (Lee and Ladd, 2001; Dash et al., 2020b; c). The amount of DNA expected from a sample is dependent on the collection state. Therefore, evidence from the same biological source but in different states will not recover the same amount of DNA. A bloodstain will typically recover 250-500 ng/cm^2 of DNA, and liquid blood will recover 20000-40000 ng/ml; or a plucked hair will recover 1-750 ng/root and a shed hair recovers 1-10 ng/root (Lee and Ladd, 2001; Cătălin et al., 2011; Dash et al., 2020b). In hair samples, nuclear DNA is found only in the follicle, while mitochondrial DNA is found in the hair shaft (Dash et al., 2020b). This indicates the importance of determining which sampling strategy to use to achieve a high success rate.

Collection Surface

Another factor in the successful recovery of DNA is the substrate. DNA can be found on various surfaces during an investigation. Surface types are categorized as flat or ridged, and porous or non-porous (Hedman et al., 2020). These primary surfaces consist of fabric, wood,

plastic, and glass. When collecting the sample, the type of DNA and the collection method will influence the success rate depending on the substrate (Brownlow et al., 2012; Vickar et al., 2018; Hefetz et al., 2019; Janssen et al., 2019).

If DNA is found on multiple substrates, it is essential to know the potential DNA recovery range for various substrates to determine the sampling priority (Daly et al., 2012). Some substrates are challenging to recover DNA, such as bricks, producing low levels of DNA. The difficulty is potentially caused by the substrates' porosity and coarse nature (Hogan et al., 2018; Vickar et al., 2018). However, the quantity of DNA recovered for wood, fabric, and glass suggests the challenge of DNA recovery for some substrates is not because of porosity (Daly et al., 2012; Dargay and Roy, 2016; Ostojic and Wurmbach, 2017; Burrill et al., 2019). One study found a significant difference in recovery between wood, fabric, and glass. Wood has a recovery range of 0 ng to 169 ng. Followed by fabric with a range of 0 ng to 14.8 ng, and lastly, glass with a range of 0 ng to 5.2 ng (Daly et al., 2012). The low recovery range for glass might be a result of the DNA binding to the substrate. DNA is known to bind to silica, the main component found in various glass materials. The binding of DNA to silica is a result of phosphate-silanol and hydrophobic interactions depending on if the DNA is single or double-stranded (Shi et al., 2015). Over a period of time, these results change. Degradation of DNA results in the success rate becoming higher for non-porous surfaces (Hogan et al., 2018).

When analyzing the recovery of touch DNA from non-porous surfaces, the success rates varied. Glass and plastic surfaces could generate a greater than 70% genetic profile. In contrast, paper surfaces could not generate that level of a genetic profile. Furthermore, most metal surfaces could not generate any genetic profile (Ostojic and Wurmbach, 2017; Wood et al., 2017; Bonsu et al., 2020). The deposit of DNA is more likely to occur through secondary transfer

(which is when deposited DNA transfers to another surface) from non-porous surfaces (Goray et al., 2010; Cale et al., 2016; Helmus et al., 2016; Kirgiz and Calloway, 2017; Pickrahn et al., 2017; Burrill et al., 2019). Goray et al. (2010) found the transfer rate of wet biological materials to increase by 52.3% when the second surface is also non-porous and 94.7% for porous surfaces. This increase in the transfer is caused by a combination of pressure and friction (Goray et al., 2010; Noël et al., 2016; Burrill et al., 2019). Thus, non-porous surfaces are an insufficient source of DNA compared to porous surfaces before degradation. Over time, however, the non-porous surfaces result in the highest success rate of a genetic profile, potentially due to the lack of absorption as seen in porous substrates (Hogan et al., 2018).

Fabric color and type will also affect the recovery of DNA from an object. It is harder to recover DNA from darker fabrics. The extraction of the DNA from theses darker fabrics might be collecting remnants of the dye (Linacre et al., 2010; Hess and Haas, 2017). The recovery rate of DNA from natural and synthetic fabrics depends on the dye. Natural fabrics, such as blue jeans, have a slightly better recovery when the dye is darker. In comparison, synthetic fabrics have a slightly better recovery when the dye is brighter (Hess and Haas, 2017).

The DNA collected from a surface can vary in quality and quantity based on the object's location. When collecting DNA that is not visible, it is helpful for investigators to have known locations with high success rates on various items to maximize efficiency. However, for some surfaces, there is no trend for DNA hot spots, such as eyeglasses (Dziak et al., 2018). This trend may be a result when a sample is either handled or worn extensively over the entire sample, where DNA can easily transfer. For instance, when collecting DNA from T-shirts, the preferred sampling areas are on the inside collar. While for gloves, the preferred sampling areas will depend on the glove material; for fabric gloves, the preferred areas are the inside surface of all

fingers or the thumb pad area, and for latex gloves, the entire inside surface area should be collected (Barash et al., 2010; Dziak et al., 2018). This same logic can be applied to a victim. Zuidberg et al. (2019) conducted a study to create a heat map for locating the offender's DNA on the victim's body and found a pattern between the offender's DNA and the victim. When the victim is heavier than the offender, the preferred sampling areas are the legs and ankles. When the weight is similar between the two or the offender is heavier, the preferred sampling areas are the arms (Zuidberg et al., 2019). Therefore, the location and surface type need to be considered together because they will influence DNA's success rate.

Collection Method and Material

A variety of DNA collection materials and techniques exist that are utilized at crime scenes: swab, tape, FTA card, scraping, cutting, and vacuum. It is essential to understand the material and technique used to collect the DNA will impact the success rate. The combination of the technique and antimicrobial agents need to be considered before collecting the DNA evidence. For instance, one study found that 4n6FLOQ swabs are incompatible with DNA IQ Lysis buffer while showing a high DNA recovery increase with Prepfiler (Dadhania et al., 2013). Another study observed a high DNA recovery on glass and metal when swabbing with NaCl 0.9% solution (Oliveira et al., 2015). Joel et al. (2015) found that the extraction protocol impacts Scenesafe FAST™ mini tape. Therefore, exactly how a sample is collected and processed is an important step in the collection process.

Swab

Buccal swabs are the most common collection method for genetic evidence, especially for non-porous surfaces (Adamowicz et al., 2014; Hytinen et al., 2017; Comte et al., 2019).

When collecting genetic evidence with a swab, the type of buccal swab needs to be considered. A range of materials are used for swabs using different designs between the materials to collect the DNA: foam, microfiber, flocked nylon, and cotton (Brownlow et al., 2012; Plaza et al., 2016; Ambers et al., 2018). Differences between the materials lie in the material's structural design and will determine the genetic material's absorption rate and location. These differences will impact the success rate of collecting various genetic evidence.

There are two techniques used to collect genetic evidence at a crime scene: single and double swab. The single swabbing technique consists of a single wet swab, while the double swab technique consists of a wet swab followed by a dry swab (Hedman et al., 2020). Since the double swab technique was introduced in 1997, it has become the preferred method (Sweet et al., 1997; Hedman et al., 2020). The technique was first recommended for recovering saliva from the skin as the method had a higher recovery rate than the classic single swab method (Sweet et al., 1997). This recommendation did not indicate that the new method would provide a higher recovery rate for collecting other genetic evidence sources, such as touch DNA. However, validation to provide evidence of the technique's effectiveness was not completed until 2020, two decades after the technique started being employed (Hedman et al., 2020).

Nevertheless, many studies and practitioners still use the double swab technique (Bright and Petricevic, 2004; Esslinger et al., 2004; Anzai-Kanto et al., 2005). Prior to the validation study, other studies using the double swab technique reported that the method improved the recovery of DNA (Sweet and Hildebrand, 1999; Pang and Cheung, 2007). The validation study does not support these recommendations completely.

The double swab technique's validation study results support the importance of selecting the proper material and method when collecting DNA. Different surfaces were tested. In general,

the classic single swab technique is better for collecting DNA, unless the surface is possibly complex consisting of both ridged and porous features, such as wood. The success rate of the second swab was tested by examining a wet and a dry swab. The results indicated that when using a second swab, a wet swab gives a higher yield. However, the second swab still provides a lower yield than the first swab (Hedman et al., 2020). Another study detected additional DNA on a non-porous surface when using the single swab method after an initial fingerprint adhesive lift recovered less than half the DNA present (Hytinen et al., 2017).

Tape

Adhesive tape is a common collection method for genetic evidence (Zech et al., 2012; Hanson and Ballantyne, 2013; Plaza et al., 2016). When collecting genetic evidence with tape, the adhesive type needs to be considered because it can interfere with collection (Barash et al., 2010; Joël et al., 2015; Plaza et al., 2016). During collection, the tape can be reapplied to the surface to increase DNA potential. However, because this method is an adhesive, there is a collection threshold that, if exceeded, decreases the mean DNA recovery percentage as the tape decreases tackiness (Verdon et al., 2014; Kirgiz and Calloway, 2017). For instance, Scotch® Magic™ tape's threshold is 8 tapings, and Scenesafe FAST™ is 32 tapings (Verdon et al., 2014). Another study found tape recovered mixed profiles in 61% of the samples. This was significantly higher than the cutting method for both a large and small volume sample (Gunnarsson et al., 2010). Therefore, it is crucial to understand the benefits of an adhesive before collection.

When is an adhesive tape, the preferable collection method? Adhesives are more suitable for recovering epithelial cells (Bright and Petricevic, 2004; Hall and Fairley, 2004; Barash et al., 2010). In comparison to swabs, adhesive tape collects more DNA from textiles. This could result from the porous surface of most textiles; however, the tape also outperforms swabs on raincoats,

which is a non-porous textile, but not on flannelette because of the loose fibers (Verdon et al., 2014; Hess and Haas, 2017). In other instances, there is no statistically significant difference between the methods of swabbing or tape lifting from a surface, such as steering wheels (Kirgiz and Calloway, 2017). The use of adhesive tape was outperformed by direct cutting for porous substrates and vacuum for non-porous substrates. However, when needing to collect from a large surface area, the adhesive method can be beneficial (Dong et al., 2017). Thus, it is essential to consider the DNA source and the collection surface before selecting adhesive tape.

Cutting

The third most common collection method is cutting. This is a destructive method; therefore, it should be used sparingly. Cutting evidence with touch DNA without knowing the key places to collect can lead to contamination or DNA loss (Dong et al., 2017). The risk of a mixture of different trace DNA increases when using this method because both sides of the sample are processed together (Sessa et al., 2019). Studies indicate no significant difference between cutouts or swabs for various textile samples, such as fabric gloves, footwear, or brassiere. However, cutouts are significant for the band on beanie-style hats (Dong et al., 2017; Dziak et al., 2018; Sessa et al., 2019). Therefore, the cutting method provides similar results to less destructive collection methods and should be used when those methods fail or insufficient.

Newer and less frequent methods

Materials and techniques other than swabs and adhesive tape are also used to collect biological evidence: FTA cards, scraping, and vacuum. However, these are less frequently used, and the research of these materials is scarce compared to swabs and tape. Because these methods are less common, the person collecting the biological evidence should be trained on the specific sampling strategy's proper techniques. These alternative methods could be beneficial for

collection when conventional methods fail or are insufficient. The vacuum method demonstrates the potential of collecting a higher DNA yield of bricks, which is a difficult surface for the traditional collection methods (van Oorschot et al., 2010; Vickar et al., 2018). This method was the best for non-porous substrates compared to swabs, direct cutting, and adhesive tape (Dong et al., 2017). Another method, known as the scraping method, demonstrates a higher DNA yield from porous textiles than swabs and occasionally adhesive tape (Hess and Haas, 2017). In an instance where the collection surface contains the victim's and offender's DNA, such as the victim's nails, soaking clippings in digestion buffer results in a higher DNA yield than swabbing or scraping (Hebda et al., 2014). However, these methods are potentially less beneficial if the cost is the primary concern (Dong et al., 2017).

FTA cards are a material that may have technical challenges if the person is unaware of the difficulties of working with this type of sample collection method. The FTA's composition helps preserve the DNA, which is beneficial for evidence storage (Kirgiz and Calloway, 2017). The FTA is made of a paper that contains an indicating circle that often changes color and will lyse the eukaryotic cells when a sample is introduced (Milne et al., 2006; Dash et al., 2020d). The DNA will bind to the FTA card after drying for 5 to 10 minutes (de Vargas Wolfgramm et al., 2009). Two collection methods that can be used to add DNA to the cards. The first is a direct application to the card where the sample must be a liquid (Milne et al., 2006; GE Healthcare, 2010; Dash et al., 2020d). The second is a scrapping method where the card is moistened, then scraped across the collection surface (Pizzamiglio et al., 2004; Kirgiz and Calloway, 2017; Janssen et al., 2019). The second method's challenge is the potential of the loss of DNA on a rough surface (Kirgiz and Calloway, 2017). Studies have found the cards are more reliable than buccal swabs by providing a statistically significant higher DNA yield, potentially due to the

card's chemical composition (Milne et al., 2006; Kirgiz and Calloway, 2017; Janssen et al., 2019).

Conclusion

With the advancements in forensic genetics, DNA as evidence is expanding because DNA can be collected from a variety of evidence sources. Nevertheless, the information learned from DNA is beneficial only if the genetic evidence is collected effectively and efficiently. This is why it is crucial to determine the best sampling strategy to achieve the highest success rate possible. DNA cannot be retroactively fixed, especially in the absence of a reference sample. Thus, the collection process is one of the most important steps when collecting genetic evidence during an investigation because a sampling strategy is not the same for all genetic evidence. Investigators need to consider the four elements for the collection process to be beneficial to the investigation. Without the knowledge of proper sampling strategies and training increases the risk of a low success rate.

The law enforcement agencies may not have the funding to select the best sampling strategy for specific genetic evidence. The cost of various collection materials, such as FTA cards, can be expensive if the method's use is less frequent. Suppose there is a cost-effective method with a similar success rate to the best sampling strategy; in that case, the investigators should use the cost-effective method because the goal is to recover useful, quality DNA. It would also be more cost-effective to increase training for the collection process because a potentially useless low DNA success rate for an investigation wastes the agency's funding.

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Chapter 4: DNA Preservation and Movement

Compared to other aspects of forensic genetics, the protocols for DNA preservation have gone through minimal changes since their inception. The protocols consistently cover two aspects: storage length and temperature. However, the storage length and temperature are in relation to each other. Protocols are created to increase evidence integrity; however, they can vary and that can cause inconsistencies. The preservation of genetic evidence is crucial in maintaining the integrity of the DNA because it can be a vital piece of information in a court of law. Therefore, it is critical to use the proper preservation methods because natural factors potentially affect the success rate of DNA recovery. This chapter explores the environmental effects and storage methods to understand the best preservation for DNA. This is then related to DNA leaching and the theoretical movement of DNA through packaging based on migration modeling.

Environmental factors can cause damage to the DNA, and this degradation is often what is discussed with the quality and quantity of forensic DNA, which can impact the integrity of the DNA. Yet, damage can occur beyond degradation that can impact the integrity of the DNA. These damages include hydrolytic reaction, oxidation, and radiation.

Hydrolytic Reaction

Also known as hydrolysis, hydrolytic reactions encompass two reactions: deamination and base loss from the 2'-deoxyribose backbone. First, hydrolytic deamination occurs where cytosine transforms to uracil, adenine transforms to hypoxanthine (6-oxy purine), and guanine transforms to xanthine (2-oxy-6-oxy purine). However, hydrolytic deamination primarily occurs to the cytosines. Secondly, hydrolytic base loss primarily occurs through depurination with the glycosidic base-sugar bond as the main target (Alaeddini et al., 2010; Marrone and Ballantyne,

2010). Therefore, the underlying cause of hydrolysis is the loss of amino groups and the effects change the coding potential on the DNA template which causes PCR miscoding lesions (Alaeddini et al., 2010). A study analyzed the process of hydrolysis on DNA in both hydrated and dried states, which found there to be the same systematic process between both DNA states. However, the dried state of DNA offers more protection to the effects of hydrolysis (Marrone and Ballantyne, 2010). In addition, both water and heat can cause this type of DNA damage to occur (Bonnet et al., 2009; Asari et al., 2018; Chauhan, 2020).

Oxidation

Reactive oxygen species result in the oxidation of DNA, which causes degradation. One of these species includes hydroxyl radicals. The presence of water can cause oxidation to occur because of the hydrogen and oxygen that forms the molecule (Bonnet et al., 2009; Tan et al., 2021). In addition, high temperatures can also cause oxidation to cause DNA damage (Bonnet et al., 2009; Asari et al., 2018; Chauhan, 2020). In the absence of reactive oxygen species, the thermal stability of DNA is higher at room temperature (Paunescu et al., 2013; Tan et al., 2021). The underlying cause of oxidation is the bacterial metabolism and radiation (Richter et al., 1988; Alaeddini et al., 2010). This then effects the base or sugar fragmentation of the DNA template which then causes PCR failure (Alaeddini et al., 2010). Oxidation will also contribute to chain breaks, base modification, and the formation of abasic sites which is a region in DNA where purine and pyrimidine bases are absent leaving just the DNA backbone (Talpaert-Borlè, 1987; Bonnet et al., 2009; Tan et al., 2021).

Radiation

Solar radiation produces three types of ultraviolet rays that transmit towards the earth's surface: UVA, UVB, and UVC. The least energetic rays are UVA rays that range from 320 to

400 nanometers that transmit 95% of the ultraviolet rays that transmit to the earth's surface. The other five percent is transmitted by UVB rays that range from 290 to 320 nanometers. The UVC rays that range from 100 to 290 nanometers do not reach the earth's surface (Hall et al., 2014). Both UVB and UVC can be absorbed by DNA, which leads to damages (Gršković et al., 2013). However, UV rays may not be the primary cause of DNA damage as water absorbs UVA and UVB rays which can produce reactive oxygen species (Hall et al., 2014).The DNA damage seen with exposure to UV rays include: base modification, strand breaks, photoproducts, oxidative damages, cross-linking, and dimer formation (Alaeddini et al., 2010; Hall et al., 2014; Tan et al., 2021). Allele drop-out only occurred after exposure to the equivalent of 795 days of UVB rays (Hall et al., 2014). The damage is primarily found in the repetitive sequences of DNA (Barlev and Sen, 2018). Yet, the use of radiation can be beneficial. As UVC rays can be used to reverse the formation of cyclobutadipyrimidine by splitting the forming cyclobutene rings (Ravanat et al., 2001). The exposure of high doses of UVC rays have also cause a rapid DNA degradation after exposure, making this form of radiation for laboratories to use for decontamination purposes. However, UVC more easily degraded longer fragmented DNA than short fragments (Gršković et al., 2013).

Storage Environments: Effects

Time was thought to affect the integrity of DNA; however, research found that the greatest effect on the integrity of DNA is environmental elements (Alketbi and Goodwin, 2019). Therefore, it is important to use the appropriate storage methods for genetic evidence because of the known effects various environmental elements can have on the DNA. Research has demonstrated that genetic degradation will occur from environmental factors, which includes

water and temperature (Lee et al., 2012; Sirker et al., 2016; Asari et al., 2018; Hakim et al., 2020; Ip et al., 2021).

Water

Humidity refers to the concentration of water vapor in the air. The level of moisture held in the air is dependent on the temperature; this is known as relative humidity (Lawrence, 2005). How does humidity potentially damage genetic evidence? Water is known to cause DNA damage, and since humidity is a percentage of water in a gaseous state, it introduces the risk of hydrolytic damage, oxidation, and molecular mobility (Bonnet et al., 2009; Tan et al., 2021). Thus, it is important to dry genetic evidence before placing it into storage when possible. For example, at 50 percent relative humidity, two things begin to occur: first, the rehydration of DNA if exposed to air, and second, the start of denaturation of DNA (Bonnet et al., 2009; Colotte et al., 2011; Tan et al., 2021). In one study, three environmental conditions were tested: 25°C with relative humidity less than 30 percent, and 25°C and 40°C with a relative humidity greater than 80 percent. The study found that high levels of humidity accelerate the degradation of DNA, and after 6 months, the degradation ratio 129:41-bp (129 base pair DNA fragment to 41 base pair DNA fragment) had significantly decreased at 25°C with 80 percent RH (Asari et al., 2018). Another study found DNA degradation after 90 days in storage with a 50 percent relative humidity (Brogna et al., 2020). The length of the DNA fragments and the sample type will affect the survival rate of DNA in humid environmental conditions. For instance, blood stains are not significantly impacted by humidity up to 93 percent relative humidity possibly due to the DNA still being encapsulated in the nucleus (Dissing et al., 2010). The use of a stabilizing agent, that protects the cellular structure, can prolong DNA integrity even in high humidity; however, this is dependent on the agent used (Lee et al., 2012; Shewale and Liu, 2013).

Under inadequate environmental conditions, the presence of water will allow the growth of microbials, which interferes with the extraction and the stability of DNA. Humid conditions have been shown to be the primary cause for the growth of microbials, which prevents little to no human DNA from surviving because of the microbial exoenzyme activity interacting with water (Dissing et al., 2010; Sirker et al., 2016; Al-munim and Al-rashedi, 2021). In one study, microbial growth began as early as a month and a half with the environmental conditions of 35°C at 100 percent relative humidity (Dissing et al., 2010).

Additionally, environmental conditions affect DNA prior to collection. These effects should be applied to the current understandings of DNA storage. A study found that high levels of humidity have different effects depending on whether the collection surface is porous or nonporous, and more DNA was collected from glass and stainless steel than porous surfaces in high humidity (Alketbi and Goodwin, 2019). Forensic evidence can be collected using various materials, which are then placed into a collection container. For example, there are swabs with plastic collection tubes and others that require a collection cardboard box. If the swabs are not dried completely, then the cardboard box could lead absorption or leaking of the sample. The study also found that moisture may increase the DNA transfer rate (Alketbi and Goodwin, 2019). Thus, it is important to ensure genetic evidence is dried.

Temperature

Studies analyzing various environments have shown that temperature plays an essential role in the degradation of DNA. The rate that degradation occurs is dependent on the temperature; for instance, DNA degrades faster at room temperature compared to refrigeration or freezer, and begins to degrade immediately in extremely hot temperatures (Frippiat and Noel, 2014; Hara et al., 2016; Ng et al., 2018; Abdel Hady et al., 2021; Al-munim and Al-rashedi,

2021). The temperature range of -20˚C to 37˚C produce the highest recovered concentration of DNA after exposure to these temperatures for twenty-four hours (Raina et al., 2004; Abdel Hady et al., 2021). However, the type of sample may determine how temperature affects the DNA because various studies have come to different conclusions on the effects of high temperatures. For instance, higher temperatures has been found to cause chain-breaking oxidative and hydrolytic reaction to degrade the DNA (Bonnet et al., 2009; Asari et al., 2018; Chauhan, 2020). Yet, another study found higher temperatures have less of an effect on the quality of the DNA (Abdel Hady et al., 2021).

Despite the freezer degrading DNA slower than other temperatures, the freeze-thaw cycle does have an impact on the integrity of DNA (Corradini et al., 2019). Exposure to multiple freeze-thaw cycles can reduce the DNA integrity, which might be a result of the formation of ice crystals during freezing (Lee et al., 2013; Tan et al., 2021). There are significant changes in the quantity of DNA during some of the freeze-thaw cycles, however, the percentage was within an acceptable range of ± 5% (Safarikova et al., 2021). There are conflicting conclusions on the rate at which a sample should be frozen. A more recent study found rapid freezing is less harmful than slower freezing of DNA (Anchordoquy and Molina, 2007; Fabre et al., 2017; Tan et al., 2021). Another study found the freezer to be an acceptable storage method for long term storage despite the freeze-thaw cycle. Rather, liquid nitrogen is unsuitable for long term storage as it causes DNA to clump (Safarikova et al., 2021).

Storage Methods

Throughout the investigation process forensic evidence will be stored for various reasons. The first step in the preservation of genetic evidence begins with the collection process. This is

typically done by crime scene investigators or police officers (Pickrahn et al., 2017). These individuals are not always trained and many investigators still depend on experience from prior casework to collect DNA (Baechler, 2016). A 2017 study found 67.1% of the contaminated samples originated from the investigators; while another noticed an increase of contamination over the years (Kloosterman et al., 2014; Pickrahn et al., 2017). After collection, evidence is often shipped to a forensic facility through various methods, such as UPS, FedEx, or USPS. During transport, packages can be exposed to extreme temperatures or repeated freeze-thaw cycle (Shikama, 1965; Davis et al., 2000; Howlett et al., 2014). Therefore, it is vital to ensure the integrity of genetic evidence throughout the transport and storage process. Optimal storage conditions prevent the exposure to water and oxygen (Bonnet et al., 2009; Shewale and Liu, 2013). Currently, there are two standard protocols that the majority of laboratories follow pertaining to time and temperature that are discussed further below. However, the protocols do not provide the necessary information for the collection of genetic evidence. For instance, protocols state evidence should be dried prior to packaging, but no further information is provided than that simple fact. Nevertheless, the protocols do provide adequate information on the packaging and preservation of genetic evidence (Cordray, 2010; Cătălin et al., 2011; Department of Public Safety - Texas, 2012; Ballou et al., 2013).

When evidence arrives to a laboratory, the evidence goes through internal laboratory processing procedures. Upon receiving the evidence there are a series of notes that must be recorded as a laboratory receipt of evidence. These notes include: type of examination request, inventory sheet, signs of contamination, and if the packages were properly packaged. Then the laboratory will conduct an internal processing procedure, which is for the laboratories records (Lee and Ladd, 2001). After these steps are completed, the evidence will move into storage.

Time

Forensic biological evidence is stored during various phases of the investigation process. The storage conditions are dependent on the stage of the investigation. Short term storage, which is sometimes rereferred to as temporary storage, pertains to any biological evidence storage under a designated length of time. According to the National Institute of Justice and the National Institute of Standards and Technology evidence may be stored in short term conditions for less than 72 hours. The environmental guidelines for evidence stored in temporary storage will differ from long term storage. While long term storage pertains to any biological evidence storage beyond that of the short term storage. The National Institute of Justice recommends long term storage conditions when storing biological evidence for more than 72 hours (Ballou et al., 2013). Despite these guidelines, due to the backlog of evidence extractions, evidence is held in storage until the evidence is extracted.

A backlog in the crime laboratory caused by low funding, low staffing, or evidence quantity, leads evidence to be held longer in storage. Evidence is potentially considered backlogged at varying rates based on the type of evidence. For instance, sexual assault kits are generally considered backlogged between 30 and 90 days after being placed into storage. However, there is no standard time frame for when evidence begins to be considered backlogged (Nelson, 2010; Quinlan, 2020). In 1997, 70% of the laboratories reported backlog, which then increased to 80% by 2000. By 2005, the average backlog request per lab increased from 86 to 152 requests by the end of the year for DNA analysis alone (Durose, 2008; Strom and Hickman, 2010). In the United States between 2011 and 2017 there has been an increase in the number of backlogged DNA requests at the end of each year (U.S. Government Accountability Office, 2019). A global audit of 148 forensic labs, between December 2020 and January 2021, was

conducted. The audit indicated there was a 34% net reduction of the 2020 backlogged samples. While, the audit predicts a 33% net increase of backlogged samples for 2021(ThermoFisher Scientific, 2021)**.** Therefore, more samples will be in storage for longer.

Another type of long term storage pertains to evidence retention of DNA from the extracted evidence. It is important to retain the evidence after DNA extraction because another extraction or analysis may be required. The retention of evidence will more likely held in police custody, while the forensic genetics laboratories will retain the extracted samples. Currently, in England and Wales the retention period is dependent of the national policy and the agencies retain the evidence. For example, the NPCC V2.1 policies suggests police and forensic services retain evidence of serious crime for six years; while Forensics 21 policy suggests forensic services should retain serious crimes for seven years (McCartney and Shorter, 2020). In Ohio, if a conviction occurs for a SB 77 crime, then the evidence must be retained for either thirty years or the latest period the convicted is in custody (Cordray, 2010). In Texas, the retention for unsolved cases is no less than forty years or until the applicable statute of limitations expires. For convicted cases, the evidence should be retained for as long as the convicted is in custody (Department of Public Safety - Texas, 2012). Therefore, the evidence retention length is dependent on the local government's laws.

Temperature

Currently, there are four temperatures evidence can be stored at: frozen (at or below -10˚C), refrigerated (between 2˚C and 8˚C, less than 25% humidity), room temperature (ambient temperature), or temperature controlled (between 15.5˚C and 24˚C, less than 60% humidity). The optimal storage temperature is dependent on the evidence type and the length of storage (Ballou

et al., 2013). It is important to maintain the temperature and humidity levels based on the storage environment and to monitor the levels weekly.

The most common temperature for DNA extracts requires many freezers and generators, which are rather expensive to maintain (Howlett et al., 2014; Corradini et al., 2019; Tan et al., 2021). According to the National Institute of Justice and the National Institute of Standards and Technology the freezer is best suited for wet type samples. However, liquid blood is the only type of evidence that should never be frozen. Both feces and urine are best stored in the freezer for any length of time. While it is best to freeze wet bloody samples for short term storage and liquid DNA extracts for long term storage. In addition, it is acceptable to freeze bones for short term storage (Ballou et al., 2013; Dash et al., 2020c).

According to the National Institute of Justice and the National Institute of Standards and Technology, refrigeration is not frequently recommended for long term storage. Refrigeration is more suitable for short term storage for many types of evidence including liquid blood and wet swabs. While it is considered acceptable to store wet bloody items in refrigeration, urine samples can only be stored at this temperature for less than 24 hours. In contrast, for long term refrigeration is best for only liquid blood, however, it is considered acceptable to store liquid DNA extracts in this environment (Ballou et al., 2013; Dash et al., 2020c).

Room temperature storage is the most cost-effective. Therefore, it is often the method chosen despite being an optimal storage temperature for a select few evidence types. Three types of evidence are considered acceptable for room temperature storage: dry biological stained items, bones, and hair. While buccal swabs can be stored at room temperature, it should be for less than 24 hours. However, only the hair samples are acceptable for long term storage at room temperature (Ballou et al., 2013; Dash et al., 2020c).

According to the National Institute of Justice and the National Institute of Standards and Technology, temperature-controlled storage is the best environment for most types of evidence for any length of time. This includes dry biological stained items, hair, dried swabs, buccal swabs, and vaginal smears. Liquid blood and wet bloody items are able to be stored in temperature-controlled environment for less than 24 hours. Finally, it is acceptable to store dried DNA extracts in long term temperature controlled storage (Ballou et al., 2013; Dash et al., 2020c).

Shelving

The type of shelving used for evidence storage is dependent on both the temperature and length of storage. A forensic facility has different storage areas based on those variations. The ideal shelving should be nonporous to prevent contamination. For general storage areas, high-density shelving can be used, which is beneficial when the facility has limited space as this shelving system is often mobile allowing the shelves to become compact by eliminating excess aisle space. High-security vaults are typically reserved for storing money, weapons, or drugs (Mozayani and Fisher, 2017). The storage equipment will vary from that of long term storage. For instance, evidence can be temporarily stored in metal lockers while the samples dry. Temporary storage can include manufactured or repurposed lockers, or under rooms and closets, commercial, residential or under-the-counter refrigerators and freezers. While long term storage includes larger shelving and rooms, such as walk-in commercial refrigerators and freezers (Ballou et al., 2013).

Solution preservation

A DNA preserving agent may be added to a sample, depending on the type of evidence. In some instances, the preserving agent can preserve DNA better in the normal storage and the

optimal agent is dependent on the storage environment. Preserving agents must be void of trace metals because it causes oxidative damage to the DNA. If stored at -80˚C, then DNA must be isolated from ethanol and then transferred to an aqueous buffer (Anchordoquy and Molina, 2007). SampleMatrix® was tested during the shipment of genetic samples, and was found to not maintain DNA as well as PTFE containers that are stored at 4˚C. However, the cost of the PTFE containers in large numbers are not cost effective (Clabaugh et al., 2007; Shewale and Liu, 2013). DNAstable™ has been found to be effective in preserving extracted DNA at room temperature. However, this has been shown to be more effective on low quantity (<20 ng) DNA samples (Howlett et al., 2014). Trehalose is another preserving agent that was found to improve the recovery of dried low-quantity DNA at room temperature. However, the presence of trehalose did not have a significant impact on DNA preservation when stored at -80˚C (Shewale and Liu, 2013). Biomatrica® was found provide better preservation than trehalose (Ivanova and Kuzmina, 2013). One study tested the preservation of saliva and blood in the presence of a buffer, which was found to preserve the integrity of the DNA for both sample types. However, the study analyzed the preservation for four years for the saliva samples and four months for the blood samples. Therefore, it is unknown if the buffer is able to preserve the beyond the four months (Burrows et al., 2017, 2019). Since preserving agents are not equal in the preserving of DNA, the agent used should be selected based on environmental conditions, storage material, sample type, and if the sample can be dried.

Storage Material

Certain types of container materials are known to better preserve DNA samples during storage. When packaging collected evidence samples the National Institute of Standards and Technology recommends using evidence bags, boxes, or envelopes for general evidence

packaging, which will then be sealed with adhesive tape. These materials allow for easy storage and organization. It is preferred that the containers are breathable to prevent condensation and allow oxygen to prevent bacteria growth (Ballou et al., 2013; Mozayani and Fisher, 2017). However, through the exposure to air dried DNA can regain water, which will lead to moisture within the container (Colotte et al., 2011). Plastic packaging is it to be avoided for both inner and outer packaging (Cordray, 2010; Department of Public Safety - Texas, 2012; Ballou et al., 2013). However, many forensic swabs available today come with a plastic collection tube, but some of the collection tubes have breathable ventilation portion near the swab tip. This ventilation allows excessive moisture to diffuse out of the tube (Aditya et al., 2011; Garvin et al., 2013; Ip et al., 2021). Some of the collection tubes are constructed of polypropylene, which is a type of plastic, that is found to interfere with the preservation of DNA because it binds with DNA for an unknown reason (Gaillard and Strauss, 1998; Kline et al., 2005; Lee et al., 2012). This binding could cause the DNA to stick to the sides of a plastic tube, and thus, lead to a decrease in the obtainable DNA from an extracted sample. The DNA bound to the tube would potentially need to be reextracted.

DNA Movement and Leaching

DNA leaching is the movement of DNA, typically through soil. Different studies have observed leaching under certain environmental conditions within the soil that migrates radially out from its source (Haile et al., 2007; Andersen et al., 2012; Emmons et al., 2017; Thomas et al., 2018). The texture and structure of the soil influences DNA leaching. Research has not detected leaching in frozen sediments. Nor has DNA been detected migrating beyond the clay strata, which research theorizes to be caused by the soil structure of the clay (Hebsgaard et al., 2009;

Arnold et al., 2011; Andersen et al., 2012). Nevertheless, DNA has been detected between 10 cm to 16 cm away from its originating source (Andersen et al., 2012; Thomas et al., 2018). However, it is still not fully understood how the DNA is moving through the soil.

Currently, there is little understood of the external movement of DNA. However, when DNA is in a solid state, which is when most water has been removed, it is known to decrease molecular mobility with only small movement possible (Bonnet et al., 2009; Colotte et al., 2011). Despite the DNA being in a solid state, the environment can reactivate the DNA mobility. Water and heat enhance the mobility of amorphous solids (Byrn et al., 2001; Bonnet et al., 2009). As DNA is dehydrated it changes its form because water is important for the nucleic acid structure. Therefore, DNA's structure changes with the level of water present. The B-form structure is a right-handed double helix that consists of ten base pairs per turn that are perpendicular to the helix axis, which is the most similar structure to the original DNA model. While the A-form structure consists of eleven base pairs per turn that are displaced and inclined to the helix axis (Dickerson et al., 1982; Ghosh and Bansal, 2003). There is twice as many water molecules per nucleotide in the B-form compared to the A-form. As water levels in DNA reduces below 70% relative humidity, the structure changes to its A-form (Westhof, 1988; Bonnet et al., 2009). If a sample is not dried completely, then the effects of water, as discussed above, is detectable in closed vials if the sample volume is significantly smaller than the container volume (Ayala-Torres et al., 2000; Bonnet et al., 2009).

Despite the limited understanding on the movement of DNA, migration modeling offers the opportunity to examine known theories of the movement of substances through materials. If the model is applied with the knowledge of DNA leaching, then this could lead to theories of the movement of DNA in storage. The elements encompassed in the movement of the models

include time, temperature, and vapor pressure (Widen et al., 2004; de Fátima Poças et al., 2011; Maia et al., 2016; Brandsch, 2017; Fang and Vitrac, 2017; Li et al., 2017). According to Fickian Diffusion, which is an ideal diffusion, diffusion depends on five things: the polymer, molecule size, temperature, pressure, and concentration (Keller and Kouzes, 2017). Materials have different permeation rates, for instance metal has the lowest rate and polypropylene has a medium permeation rate. As temperature rises the permeability increases approximately five percent per degree in Celsius (Stannett and Williams, 2007; Keller and Kouzes, 2017). For instance, using the moisture vapor transmission rate (MVTR), which measures the rate water vapor passes through material, the rate for a 0.58 mm thick piece of polyvinyl toluene (PVT) in 100% humidity was 2.65 $g*m^{-2}*d^{-1}$ at 30˚C, 4.4 $g*m^{-2}*d^{-1}$ at 40˚C, and 7.19 $g*m^{-2}*d^{-1}$ at 50˚C. Therefore, permeation will occur at a faster rate in higher temperatures. To keep water from premating, the MVTR must be below 10^{-6} $g*m^{-2}*d^{-1}$ and packaging greater than 0.25 mm thick (Keller and Kouzes, 2017). Therefore, it is important to maintain low humidity and temperature levels when storing evidence samples to reduce the various permeability rates.

Conclusion

When preserving DNA there are several aspects that need to be considered before placing genetic evidence into storage. The storage environment is the most important element to consider when preserving DNA, as the environment can significantly impact the DNA because many of the effects of the environmental factors coincide. Ensuring that genetic evidence is dried completely may be the most important element in preserving DNA as water causes DNA damage, microbial growth, and the rehydration at a relative humidity of fifty percent. The preservation of DNA must begin during the collection process since the materials used to collect

the DNA will influence the preservation of the sample. Since evidence can be in storage for an extended period of time it is important that the preservation methods are available in detail for law enforcement. In addition, the knowledge of migration models provides insight into how DNA potentially moves and how molecules can move through packaging material. Thus, the rehydration of DNA potentially allows it to permeate through layers of packaging.

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Chapter 5: Methods

The methods of this research expand upon the preliminary study by focusing on the potential reasoning behind cross-contamination (Ramey, 2019). This chapter outlines the preliminary study, hypotheses, and methods of this project. The samples used for testing were either exposed to male salvia or remain unopened, then placed into storage then extracted, followed by Qubit®, qPCR, and Y-STR analysis to determine if contamination is present in the blank swab samples.

The preliminary study for this research tested to see if contamination was possible during the storage process. Two collection materials were tested: buccal swabs and Whatman cards. Extracted pig DNA was introduced to the samples as a proxy for human DNA for the study to ensure any detected contamination was not from the researchers. The variables analyzed were sample drying time prior to storage and storage time. Contamination was detected in a significant number of samples in both types of collection materials. There was no significance in the drying time; however, contamination was observed, which could result from the reabsorption of water to air-dried DNA during storage (Colotte et al., 2011; Ramey, 2019). There was significant contamination the longer samples were in storage. Therefore, DNA is moving, but it is not understood how it moves.

Hypotheses

Hypothesis I: The likelihood of DNA migration through a material is not influenced by an increase in temperature.

Temperature is one of the elements typically controlled in forensic storage. The temperature has been found to affect the packaging material, the diffusion of molecules, and

increase the molecular motion mobility (Maia et al., 2016; Brandsch, 2017). Nevertheless, migration of molecules through materials is thought to be random; however, it is more plausible for migration at a higher temperature when the flexibility of the material and molecular mobility is higher meaning the migration will not be random (Triantafyllou et al., 2005; Wang et al., 2010, 2012; de Fátima Poças et al., 2011). An increase in temperature would demonstrate a linear rate of increased contamination with increased temperature. If the contamination is not influenced by temperature, then contamination rates will be nonlinear.

Hypothesis II: At a given temperature, neither vapor pressure nor humidity influences the movement of DNA.

Humidity is another of the elements typically controlled in forensic storage. The diffusion of molecules can occur in either the liquid or air phase, and the difference between the phases is the diffusion rate (de Fátima Poças et al., 2011; Maia et al., 2016; Fang and Vitrac, 2017). Evaporation will occur when the vapor pressure is greater than water vapor. Condensation will occur when the vapor pressure is less than the water vapor, which was observed during the preliminary study (Anderson, 1936; Marek and Straub, 2001; Kryukov et al., 2014; Ramey, 2019). One study found the migration rate of the molecule benzophenone increases with humidity after 30 days, which can be applied to DNA using a similar model despite benzophenone and DNA having different physical principles to migration (Barnkob and Petersen, 2013). With this hypothesis, an increase in contamination with an increase in humidity would be caused by condensation.

Materials and Methods

Survey

A survey was sent to fifty-five government and privately funded forensic laboratories across the United States. The survey was an anonymous questionnaire with seventeen questions, which helped determine the research's protocols, drying method, storage environment, and storage time for this project. The twelve responses provided insight into the process of placing evidence into storage beyond the information found within various forensic laboratory handbooks. With the survey, it was determined that room temperature storage, followed by refrigeration, are the primary temperatures forensic laboratories use for storage of evidence. How laboratories dry genetic evidence was almost evenly distributed between the answers; however, a swab dryer was the more often used method of drying. The majority of the forensic laboratories use the Qiagen EZ1 extraction kit, which requires a specific automatic machine to utilize to complete DNA extractions.

Questionnaire

1. How is biological evidence mailed to the lab? Select all that apply.
    a. UPS; FedEx; USPS; dropped off; picked up by the lab; overnight; express; first-class; priority; standard; other
2. How long does it take biological evidence from being delivered to the lab to being placed into storage prior to extraction (from point A to point B)?
    a. Less than 24 hours; less than 72 hours; a week; 2-3 weeks; a month; 2-3 months; 6 months; other
3. What temperature does your lab store genetic evidence prior to extraction?
    a. Refrigerated; room temperature; controlled room temperature; frozen

4. What regulation protocol does your lab use? Check all that apply.
    a. Lab's own protocol; International Association for Property and Evidence (IAPE); The Biological Evidence Preservation Handbook (NIST and NIJ); Quality Assurance Standards for Forensic DNA Testing Laboratories; Other
5. What is the average length of time (in weeks) genetic evidence remains in storage at your lab prior to extraction?
6. Does extracted evidence go back into storage prior to analysis? If yes, what is the average length of time (in days) genetic evidence is left in storage between extraction and analysis?
7. What is the average number of swab(s) taken from evidence?
    a. 1; 2; 3; 4; 5+
8. How does your lab dry genetic evidence?
    a. Swab rack on counter; swab rack in hood; swab dryer; other
9. How is dried biological fluid evidence stored BEFORE extraction in your lab? Select all that apply.
    a. Refrigerator; freezer; evidence locker; high density mobile shelving; open shelving; other
10. How is dried biological fluid evidence stored AFTER extraction in your lab? Select all that apply.
    a. Refrigerator; freezer; evidence locker; high density mobile shelving; open shelving; other
11. How is extracted evidence stored long term. Select all that apply.

a. Large plastic container; large cardboard container; medium plastic container; medium cardboard container; small plastic container; small cardboard container; large evidence envelope; large glassine envelope; medium evidence envelope; medium glassine envelope; small evidence envelope; small glassine envelope; evidence tape; plastic tube; evidence-pro blank security bags; other

12. Are genetic evidence samples stored in proximity to other materials, or other samples? If yes, is the genetic evidence stored in proximity to other evidence from the same case or non-related case?
    a. No; Yes, same case; Yes, non-related case
13. What is the approximate distance between genetic evidence samples? Select all that apply.
    a. Multiple samples stored together in a box; multiple samples stored together in an envelope; storage containers physically touching one another; other
14. What DNA extraction protocol does your lab use? Write in Answer.
15. Do you ever have to re-extract samples?
    a. Frequently; occasionally; rarely; never; other
16. Does the lab randomly test for contamination of samples left in long term storage?
    a. Yes; no; other
17. How is your lab funded?
    a. Government; private; both; other

Storage Setup

The questionnaire allowed for the research methods to be refined to provide a more accurate setup based on forensic laboratories across the United States. For the project, each

tested variable consisted of two types of boxes. The first type consisted of the ten control swabs, which remained unopened until the swabs were removed from storage for extractions. The second type consisted of five blank swabs and five known swabs (Table 5.1). The blank swabs were left unopened until the swabs were removed from storage for extractions and stored in the same container as the known swabs, which were introduced to DNA prior to storage. BD BBL™ CultureSwab™ Sterile swabs were used for this study, and each swab was labeled with the sample type, reference number, and entry date, utilizing a sharpie on the paper label on the exterior.

Table 5.1. Sample types and numbers used for analysis.

Box Type	Sample Type	Number of Swabs
1: Controls	Control	10
2: Known/Blank	Blank	5
2: Known/Blank	Known	5

For this study, the saliva was collected from one male volunteer. The Institutional Review Board approved the study (Reference No.: 118-20), and informed written consent was obtained from the participant. Saliva was collected using a sterile 15 mL tube, which 50 μL would then be applied to each of the known swabs. The known swabs were then dried for twenty minutes using the Dry-Fast Swab Dryer, which had been cleaned with DNA Away on the internal and external surfaces then left to dry **(**Figure 5.1**)**. The swabs were then placed into the microcentrifuge tube rack, which was Velcroed to the bottom of the box, based on the layout displayed in figure 5.2 (appendix A,B). The blank swabs were removed from its packaging, labeled, and immediately placed in the tube rack, without having broken the paper seal, after the known swabs had been placed. This seal visually indicates if a swab has been opened and

potentially exposed to air. Before placing the samples into storage, the box, tube rack, TraceableGo™ Datalogger, and any Barska dehumidifier bags were cleaned with DNA Away and allowed to dry completely. The samples then remained in storage for 45 days before being extracted. The length of time was derived from an analysis of the survey and my prior preliminary research. Based on the survey, the most frequent average time in storage was 6 weeks. This was similar to the original 45 days used in the preliminary research (Ramey, 2019). The boxes were stored in different locations (Modern Laboratory, Ancient DNA Laboratory, antechamber, or my apartment) to ensure no cross-contamination occurred between the two boxes.

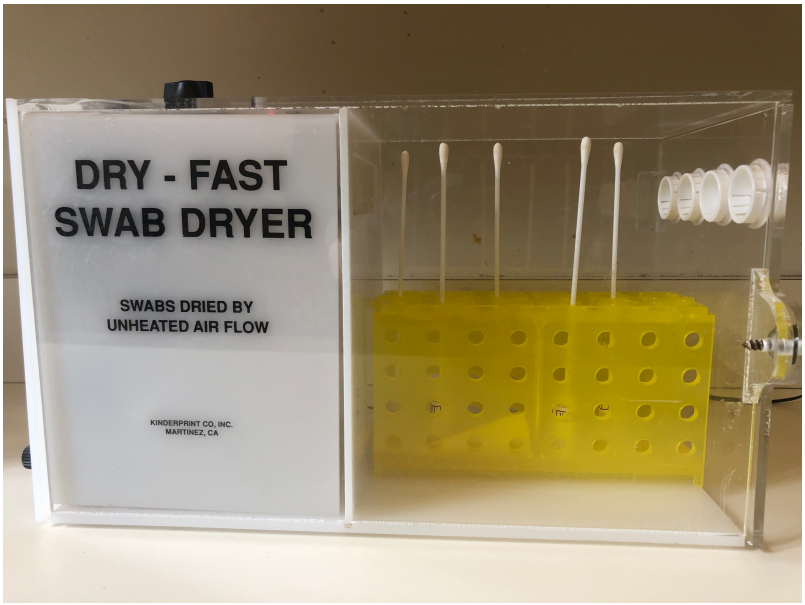

Figure 5.1. Five swabs are drying for 20 minutes in the Dry-Fast Swab Dryer after applying saliva.

X			X									X			X
						X			X						
	X			X							X			X	

Figure 5.2. The placement of the swabs during the storage process. Red indicates swabs with known DNA, and black are blank swabs.

TraceableGo Datalogger

Relative humidity and temperature levels were tracked with a TraceableGo™ Datalogger Hygrometer (Cole-Parmer Instrument Company: Traceable® Products., 2018). With the use of the hygrometer, the temperature was monitored with an accuracy of +/- 0.4 °C between -10 °C to 70 °C and with an accuracy of +/- 0.5 °C between -10 °C and -20 °C. Humidity was monitored with an accuracy of +/- 3% between 5 and 75 percent relative humidity and an accuracy of +/- 5% outside that range (Cole-Parmer Instrument Company: Traceable® Products., 2018).

The datalogger was secured within the container with magnets on one of the side walls. Four dataloggers were utilized: CC6537-6374, CC6537-6380, CC6537-6333, CC6537-6302. The dataloggers had the same configured settings for the start/stop mode, memory mode, unit preference, and logging interval. The levels were logged every hour and one minute throughout the storage process (Appendix C).

Temperature

Three temperatures were tested: room temperature, freezer, and refrigeration (Figure 5.3-5.5). Each box contained one TraceableGo™ Datalogger Hygrometer, one Barska dehumidifier bag, and the samples. Type one boxes were stored in either the anti-chamber or the ancient DNA laboratory at the University of Montana. Type two boxes were stored in the modern laboratory at the University of Montana. The modern laboratory has a workbench table and a counter opposite

each other, which allowed separation between setup and the storage area. As indicated in storage setup, type one boxes contain the controls, while type two boxes contain the blank and known swabs (Table 5.1). The room temperature controls were stored in the antechamber that leads into the Ancient DNA Laboratory at the University of Montana. The antechamber is a small room that stores supply from the laboratories and separates the Ancient DNA Laboratory from the rest of the building. A UV light is utilized in the antechamber to eliminate DNA from highly contaminated objects. The container was wrapped in aluminum foil to ensure the UV light in the room did not interfere with the samples. Prior to storage the counter or shelving were cleaned with DNA Away.

Figure 5.3. Type two box of the room temperature samples on the modern lab counter.

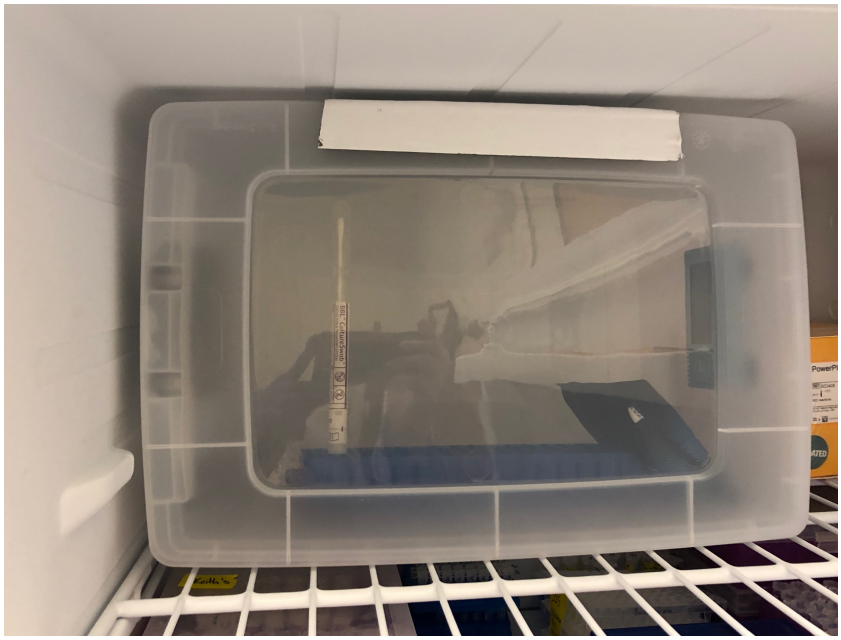

Figure 5.4. A representation of the samples in the modern lab freezer.

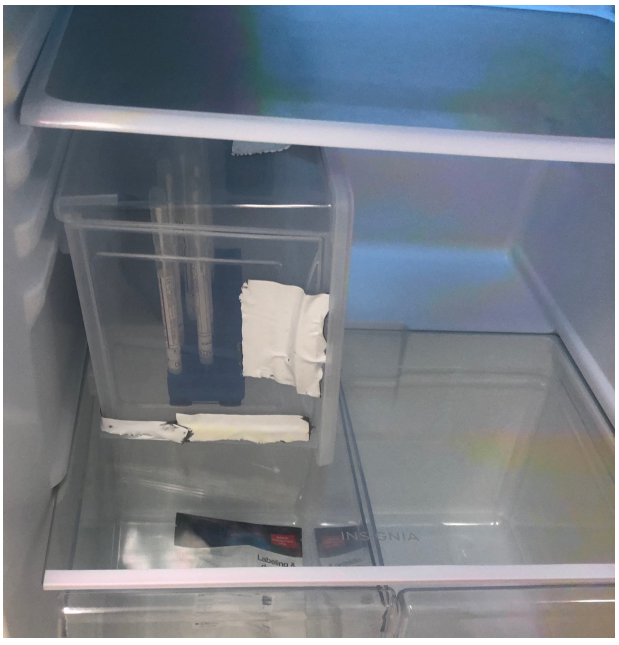

Figure 5.5. Type two box of refrigerated samples in the modern lab refrigerator.

Humidity

Three relative humidity levels were tested: approximately zero, 35%, and 50-60%. To ensure no contamination during the storage process the box types were stored in different locations. Type one boxes were stored in the anti-chamber, and type two box were stored in the

modern laboratory at the University of Montana, apart from the 50-60% relative humidity samples, which were stored in an enclosed closet in my apartment to provide access to secure Wi-Fi for a Kasa Smart Wi-Fi Plug Mini (for the humidifier) while maintaining a consistent environment. As indicated in storage setup, type one boxes contain the controls while type two boxes contain the blank and known swabs (Table 5.1). A UV light is utilized in the antechamber to eliminate DNA from highly contaminated objects. The container was wrapped in aluminum foil to ensure the UV light in the room did not interfere with the samples.

The relative humidity was monitored to achieve approximately zero humidity throughout the storage process. Each box for these samples contained two Barska dehumidifier bags (Figure 5.6). Periodically throughout the storage process, the box was opened to reset the humidity levels by microwaving the dehumidifier bags because the relative humidity levels would slowly increase. Prior to opening the box, the area around the box was cleaned with DNA Away. The box was briefly opened to obtain the dehumidifier bags to reset the humidity levels. Then the box was closed while the dehumidifier bags were microwaved for 4 minutes each. The dehumidifier bags were then wiped with DNA Away and placed on paper towels to cool down for another 4 minutes before being placed back into the boxes. Type one box was opened once on November 9th. While type two box was opened on three occasions, October 6th, October 8th, and November 2nd.

A 25% relative humidity set of samples was going to be conducted. However, after conducting the storage of the room temperature samples the humidity levels were at approximately 25%. The setup of the room temperature samples was the same as the humidity samples, except for the number of Barska dehumidifier bags within the box. The humidity

samples were also stored at room temperature. Therefore, these samples double as a test for the temperature and humidity variables.

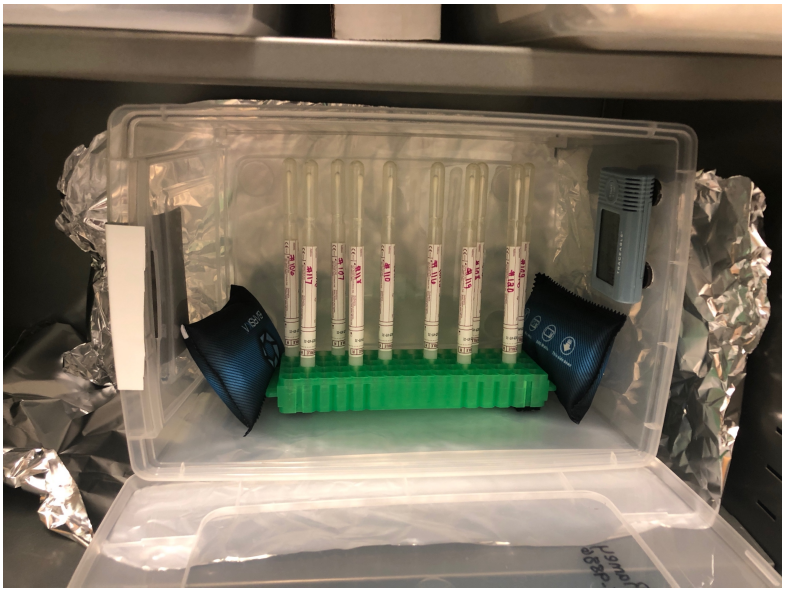

Figure 5.6. Type one box of approximately zero relative humidity in the process of being placed into storage in the anti-chamber with aluminum foil placed around the container.

Figure 5.7. Type two box of relative humidity 35% on the modern lab counter

The 35% relative humidity samples did not contain a Barska dehumidifier bag within the box. The type one box was wrapped in aluminum foil to protect from UV rays and stored in the

anti-chamber. The type two box was stored in the modern lab at the University of Montana (Figure 5.7).

The two 50-60% relative humidity boxes were stored in the same location, but at separate times, due to the setup required to achieve the humidity levels. Type one box was stored first to ensure that the environment was not the potential cause of contamination. Type one box and two of the 50-60% percent relative humidity samples were stored in an enclosed closet with an outlet, cleaned with Clorox Bleach, and lined with aluminum foil. A small slit was cut into the seal tape, coving an opening in the box, to allow the Zoo Reptile Fogger Terrarium humidifier tubing to be inserted into the box (Figure 5.8). A Kasa Smart Wi-Fi Plug Mini was used to control the humidifier throughout the storage process and allowed for more accurate control over the relative humidity (Table 5.2).

Table 5.2. The automatic on/off schedule using the Kasa Smart plug to control the humidifier

ON	OFF
12:00 AM	12:03 AM
1:36 AM	1:39 AM
3:15 AM	3:18 AM
4:51 AM	4:54 AM
6:27 AM	6:30 AM
8:06 AM	8:09 AM
9:42 AM	9:45 AM
11:18 AM	11:21 AM
12:54 PM	12:57 PM
2:34 PM	2:37 PM
4:10 PM	4:13 PM
5:46 PM	5:49 PM
7:22 PM	7:25 PM
8:57 PM	9:00 PM
10:36 PM	10:39 PM

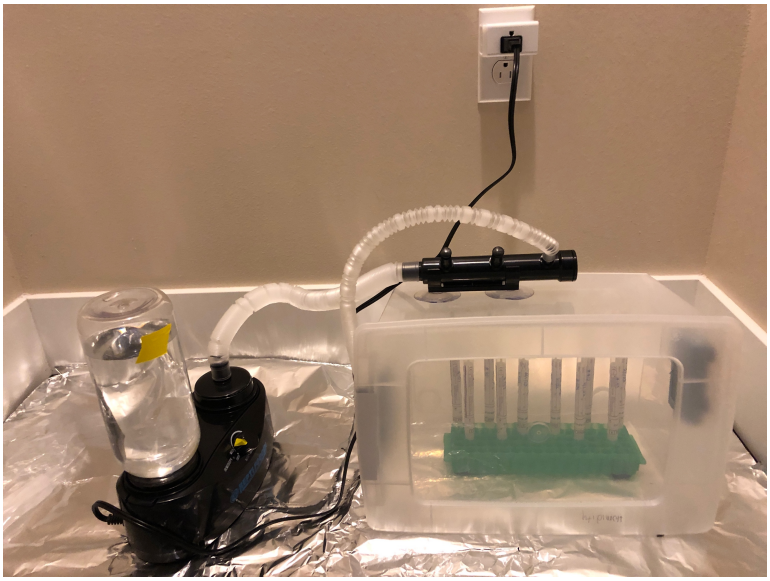

Figure 5.8. Type two box of relative humidity 50/60% in enclosed closet with aluminum foil underneath.

Extractions

Extractions were conducted in the Ancient DNA Laboratory at the University of Montana. The lab is an enclosed room with an antechamber separating the laboratory from the rest of the building. Before entering the lab, the individual must be wearing proper attire consisting of a coverall, hairnet, mask, gloves, and protective arm sleeves. The lab was wiped down with bleach and DNA Away, eliminating DNA from highly contaminated objects. In addition, between every utilization of the laboratory the overhead UV lights are activated to eliminate DNA from highly contaminated objects.

The two boxes were extracted on separate days (Table 5.3). The swabs from type one box, the controls, were extracted with two extraction control blank swabs. The swabs from type two box were split into two groups for extraction. The first group, from type two box, contained the blank swabs and an extraction control blank swab followed by the known swabs and an extraction control blank swab — the extraction control blank swab was present to try and

determine if potential contamination originated from the extraction process. Between the blank swabs and known swabs excitation, the workbench was wiped down again with DNA Away.

Table 5.3. The three extraction groups listing what box the samples originated, the sample type, and number of extraction control swabs were extracted along with the samples.

Box Type	Sample Type	Extraction Controls
1	Control	2
2	Blank	1
2	Known	1

DNA extraction from the swabs was done following a ChargeSwitch™ gDNA Buccal Cell Kit protocol (Invitrogen, Carlsbad, CA, USA). The extractions resulted in twelve separate sets. Twenty-four low binding tubes, twelve collection tubes, two 15 mL tubes, and dog nail clippers were placed into the UV crosslinker to allow UV radiation to degrade any potential contaminating DNA on their surfaces. The 15 mL tubes were removed from the UV crosslinker after 5 minutes. The low binding tubes and collection tubes were removed from the UV crosslinker right before use within the extraction process, with the first set of tubes being removed after approximately 10 minutes. The dog nail clippers were removed from the UV crosslinker after 10 minutes. A bead bath (aluminum beads to substitute for a water bath) was heated to 37°C. While the bath heated, the lysis mix and purification mix were prepared. The lysis mix contained 12 mL of ChargeSwitch® lysis buffer and 120 μL of proteinase K, that was then inverted three times. The purification mix, for twelve and a half samples, contained 500 μL of fully resuspended ChargeSwitch® magnetic beads and 1250 μL of ChargeSwitch® purification buffer.

Preparing the lysate was the first step in the extraction process. The first set of twelve low binding tubes and clippers were removed from the UV crosslinker. The tubes were then labeled on the exterior with a sharpie based on the sample number. A swab would be opened and placed with the tip side down into the corresponding tube, cutting the stem with the clippers as close to the tip as possible. The clippers were cleaned with DNA Away and then placed in the UV crosslinker for 20 seconds between each sample. Next, 1 mL of the lysis mix prepared prior was added to each tube, ensuring that the swab tip was completely immersed. The samples were then incubated in the bead bath for 20 minutes.

Binding DNA was the next step in the extraction process. Once the samples were removed from the bead bath, the supernatant was removed from the low bind tubes and placed into the corresponding collection tubes. The purification mix that was prepared was then resuspended so that the magnetic beads were evenly distributed throughout it. Then 140 μL of the mix was added to the collection tube and pipetted gently fifteen times using a 300 μL pipette. Next, the samples were incubated for a minute at room temperature. This was followed by the collection tubes being placed in a magnetic rack for a minute. Once the beads formed a pellet, the supernatant was collected and discarded without disturbing the pellet.

Washing DNA was the next step in the extraction process. With the collection tubes still in the magnetic rack, 1 mL of ChargeSwitch® washer buffer was added to the tube. The magnetic pellet should have been resuspended when the buffer was added. The tubes remained in the magnetic rack for a minute to allow the magnetic pellet to reform. Then, the supernatant was be collected and discarded without disturbing the pellet. The washing DNA steps were repeated once for a total of two washes.

The final step was eluting the DNA. The collection tubes were removed from the magnetic rack, and 150 μL of ChargeSwitch® elution buffer was added to the tube and pipetted gently 10 times. Next, the samples were incubated at room temperature for a minute. The tubes were placed back onto the magnetic rack for a minute. The supernatant was collected and placed into a new low bind, labeled tube. After extractions were finished, the lab was wiped down with bleach and DNA Away, and the UV light would be activated for an hour to eliminate DNA from highly contaminated objects.

Qubit

The Qubit® dsDNA HS Assay was used to determine if there was quantifiable DNA. The analysis provides an expected range and quality of the double stranded DNA present in the sample (Nakayama et al., 2016). The Qubit® tubes were placed into the UV crosslinker for an hour prior to use. Two standards were quantified for each group of samples. The standards consisted of 190 μL of Qubit® and 10 μL of Qubit® dsDNA HS of either standard #1 or standard #2 in a 0.5 mL thin-walled tube. Then 195 μL of Qubit® and 5 μL of the corresponding sample were added to a 0.5 mL tube for the samples. Next, each tube was vortex for 10 seconds before incubating for 2 minutes at room temperature. Following this, a Qubit4 was used to measure the concentration of DNA in each sample.

qPCR

Quantitative PCR was conducted using the Plexor® HY System kit with the Stratagene Mx3000P® instrument to quantify the DNA extract's Y-chromosome quantity. The qPCR tubes and lids were placed into the UV crosslinker for thirty minutes prior to use to allow UV radiation to degrade any potential contaminating DNA on their surfaces. While the tubes were in crosslinker, the reaction mix for quantification assays was prepared which consisted of 18 μL per

reaction plus two additional reactions to account for pipetting error. Prior to creating the mix, the Plexor® HY 2X Master Mix, Water Amplification Grade, and Plexor® HY 20X Primer/IPC Mix were thawed at room temperature and then vortexed for 10 seconds. For each sample, the mix consisted of 10 μL of Plexor® HY 2X Master Mix, 7 μL of water, and 1 μL of Plexor® HY 20X Primer/IPC Mix, that was then vortexed for 10 seconds. Next, the bench was wiped down with DNA Away. Then, 18 μL of the reaction mix was added to the qPCR tubes followed by 2 μL of the extracted samples.

The preparation of the qPCR samples were dependent on the swab type. The extracted samples were prepared before the standards and the no-template controls to reduce the chance contamination during the setup of the qPCR mix. The blank samples were done in duplicate to account for variability in pipetting, sampling, or amplification, as well as to increase the chances of potentially picking up any DNA. The control (no DNA ever in the samples or boxes) and extraction control (swabs used for to aid in detecting extraction contamination) samples were not duplicated because these samples should not have any DNA present in the samples. The known samples were also not duplicated because a high concentration of DNA was expected to be present in the samples. The extracted samples were then sealed and placed to the side.

Following the preparation of the extracted samples, the standards and no-template controls were prepared. A TE^{-4} buffer was created by combining 1 mL of Tris-HCI, 20 μL of 0.5M EDTA, and 99 mL of DI water. A serial dilution was created by thawing Plexor® HY Male Genomic DNA Standard 50 ng/ μL and then vortexed for 10 seconds. The dilution was started by adding 10 μL of the Male Genomic Standard to a 0.2 mL tube followed by 40 μL of TE^{-4} buffer to make a concentration of 10 ng/ μL. This dilution was then vortex for 10 seconds. Next, 10 μL of the 10 ng/μL concentration was added to the next 0.2 mL tube followed by 40 μL of TE^{-4}

buffer to make the 2 ng/ μL, which was then vortexed for 10 seconds. This continued another four times, with each concentration reducing by twenty percent (Table 5.4). Then, 18 μL of the reaction mix was added to the qPCR tubes followed by either 2 μL of one of the standard dilution or TE^{-4} buffer. The standards and no-template controls were done in duplicate. The unknown and standard samples were then placed into an opaque, plastic, yellow bag to protect the samples from the light as it was transported from the Ancient DNA laboratory to the Genomics Core across campus of the University of Montana.

Table 5.4. Dilution of Plexor® HY Genomic DNA Standard

Concentration	Volume of DNA	Volume of TE^{-4} Buffer
50 ng/ μL	Undiluted DNA	0 μL
10 ng/ μL	10 μL of undiluted DNA	40 μL
2 ng/ μL	10 μL of 10 ng/μL dilution	40 μL
0.4 ng/ μL	10 μL of 2 ng/μL dilution	40 μL
0.08 ng/ μL	10 μL of 0.4 ng/μL dilution	40 μL
0.016 ng/ μL	10 μL of 0.08 ng/μL dilution	40 μL
0.0032 ng/ μL	10 μL of 0.016 ng/μL dilution	40 μL

The qPCR samples were run with the Mx3000P® software using the SYBR Green (with Dissociation Curve) experiment type. In the optics configuration window, the dyes definitions were listed as name "CO560" and filter set "HEX-JOE filter set." They dye were then assigned for each filter set type. Filter set CY5 was assigned dye IC5, ROX filter was assigned dye CR610, filter HEX-JOE was assigned dye CO560, and FAM filter was assigned dye FAM. The filter gains were then set to CY5 x1, ROX x1, HEX-JOE x1, and FAM x2. Next, on the plate setup tab the wells that were being used were highlighted and designated as unknown well type. Then, the FAM, CO560, and CR610 fluorescence data were selected and the IC5 was designated as the reference dye for each well.

Continuing in the Mx3000P® software, the thermal cycling profile was set to the Plexor® kit's specifications for three segments. The first segment was the initial denaturation step that reached 95˚C for two minutes. The second segment was the denaturation step that ran for 38 cycles. The annealing phase reached 95˚C for 5 seconds followed by the extension phase that decreased to 60˚C for 40 seconds. During the extension phase endpoint data was collected at two points. The third segment was the melt temperature curve step that ran for 48 cycles. The initial temperature for this segment was 65˚C with a 0.6˚C increase each cycle for 40 seconds. Endpoint data was collected at two points during the third segment.

The qPCR results were then analyzed using the Plexor® Analysis Software. The data was imported as a new run with the Stratagene Mx3000P® selected and the autosomal target assigned the FAM dye for amplification and melt, Y target assigned C0560 for amplification and melt, IPC target assigned CR610 dye for amplification and melt, and passive reference target assigned IC5 dye. The wells containing the extracted samples were selected and defined as unknown. The wells containing TE^{-4} buffer were selected and defined as no-template control. The wells containing standards were selected and assigned the specific concentration of DNA. After this was completed, the melt threshold temperature of the correct amplicon was adjusted to the expected target melt temperature based on the specific dye type: FAM target range 79-81˚C, CO560 target range 81-83˚C, and CR610 target range 79-81˚C. The d(RFU)/dT, which measures the change in fluorescence in relation to temperature, was set to the default threshold of 25 percent. This threshold is calculated and based on the standard samples used in a single qPCR analysis. Then, a standard curve was generated by selecting all the samples to determine the DNA concentration of the unknown samples. The standard curve calculated the concentration of DNA present in the sample, which was used to determine the samples that required an STR

analysis. Due to low concentration of DNA, an additional melt curve threshold was analyzed to determine if Y-chromosomal DNA was present in the sample at a lower threshold, which was set at 5 percent.

STRs

STR analysis was conducted using PowerPlex® Fusion System with the Applied Biosystems® instrument to corroborate the results from the qPCR analyses and establish the genetic identity of the DNA located in the non-DNA exposed samples. The PowerPlex® Fusion 5X Master Mix, PowerPlex® Fusion 5X Primer Pair Mix, and Water Amplification Grade were thawed and vortexed for 15 seconds. After the samples thawed, the PCR amplification mix was prepared which consisted of 10 μL per reaction. The mix consisted of 5 μL of PowerPlex® Fusion 5X Master Mix and 5 μL PowerPlex Fusion 5X Primer Mix. The amplification mix was then vortexed for 10 seconds. Next, 10 μL of the amplification mix was added to each well. Then 15 μL of extracted DNA was added to the corresponding well. For the positive amplification control it was vortexed for 10 seconds and diluted to 0.5ng.

Using a 96-well thermocycler (ThermoFisher) the cycle was set based on the Plexor® Fusion kit's specifications for four segments. The first segment reached 96˚C for one minute that ran for one cycle. The second segment contained three plateaus: the first reached 94˚C for 10 seconds, the second decreased to 59˚C for one minute, and the third reached 72˚C for 30 seconds. The second segment ran for 32 cycles. The third segment decreased to 60˚C for 10 minutes, which ran for one cycle. The fourth segment decreased to 4˚C on a hold.

Once the thermal cycle completed the fragment analysis was completed. A loading mix was prepared by combining 1 μL of internal lane standard and 9.5 μL formamide per sample. The loading mix was then vortexed for 15 seconds. Then, 10 μL of the loading mix and 1 μL of

amplified sample was added to each well. The wells were then briefly centrifuged. Next, the samples were denatured at 95˚C for 3 minutes followed by a freezer plate block bath for 3 minutes before being placed into the ABI 3031 instrument in the UM Genomics Core. Then, analysis was conducted using GeneMapper version 3.6.

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Chapter 6: Results

Questionnaire Survey

The questionnaire was sent to different forensic facilities across the United States, twelve of the laboratories that were contacted responded to the survey, of which 75% were government funded. The responses to the questionnaire help develop the research and determine what areas to focus on within the storage process. There were a series of six questions on the questionnaire that pertained to operations of the DNA analysis facilities. The Quality Assurance Standards for Forensic DNA Testing Laboratories was utilized by 54.5% of the facilities (Figure 6.1). When asked what DNA extraction protocols the facility uses, 75% responded with Qiagen EZ1 protocol (Figure 6.2). The drying method varied amongst the facilities; however, the swab dryer was the most frequent method at 33.3% (Figure 6.3). Facilities rarely re-extract samples or test for contamination after the initial evidence analysis (Figure 6.4-6.5).

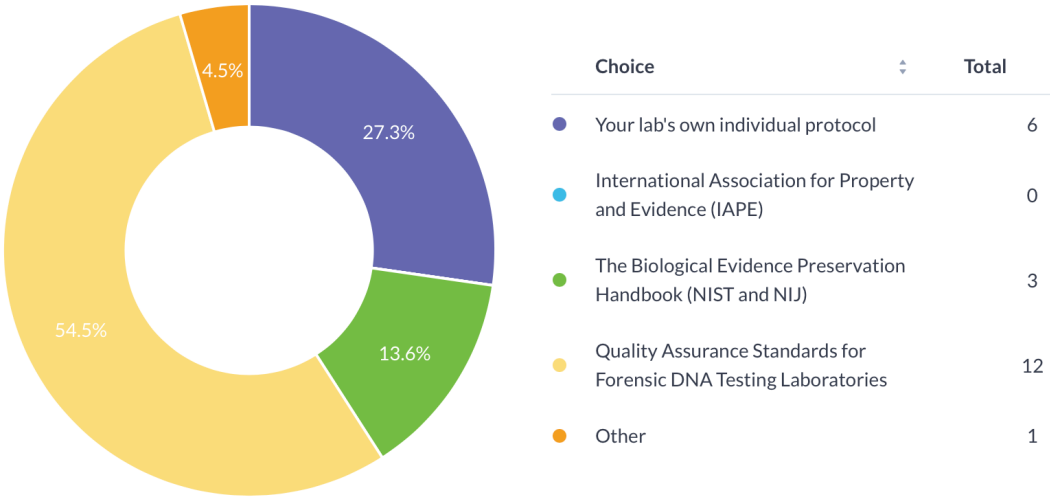


Figure 6.9. What regulation protocol does your lab use? Check all that apply. Multiple Choice.

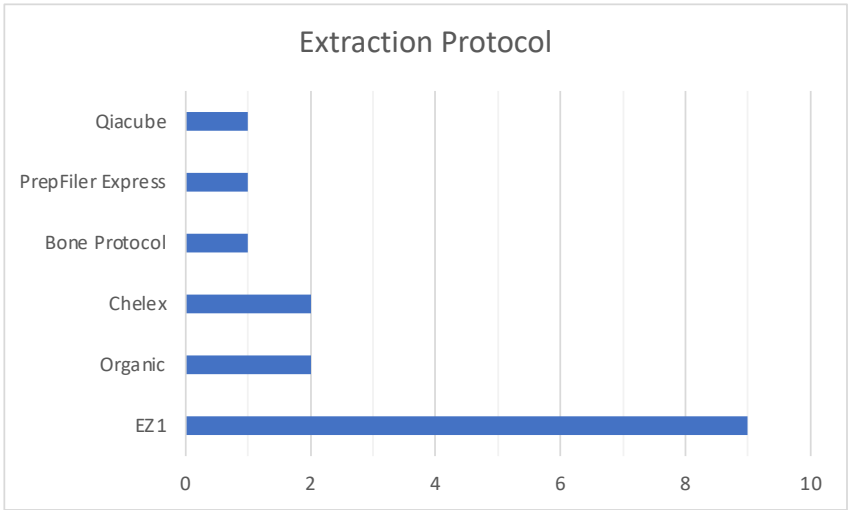


Figure 6.10. What DNA extraction protocol does your lab use? Open response.

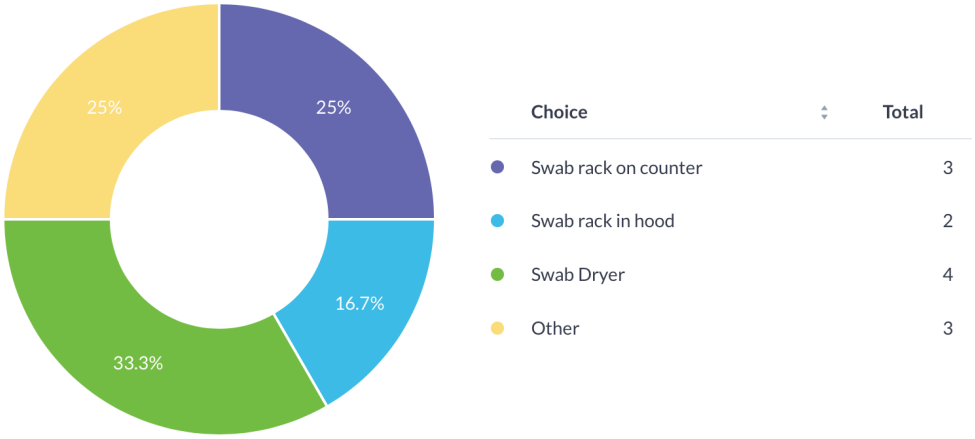


Figure 6.11. How does your lab dry genetic evidence? Multiple Choice.

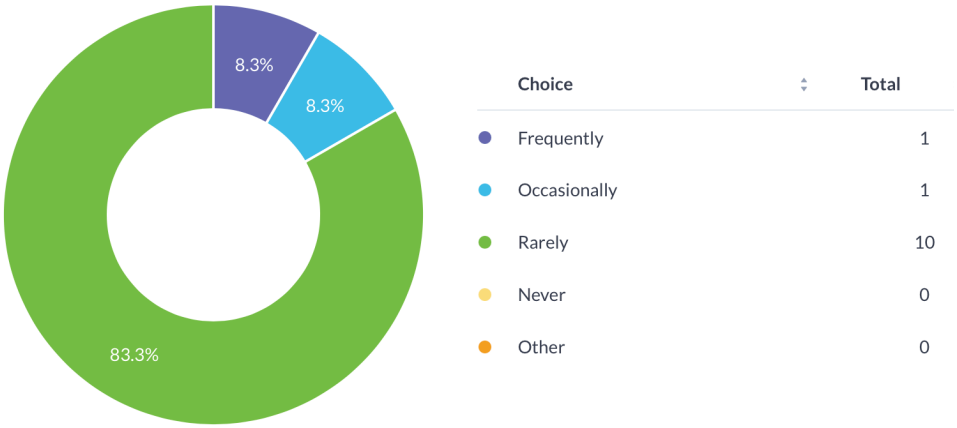


Figure 6.12. Do you ever have to re-extract samples? Multiple Choice.

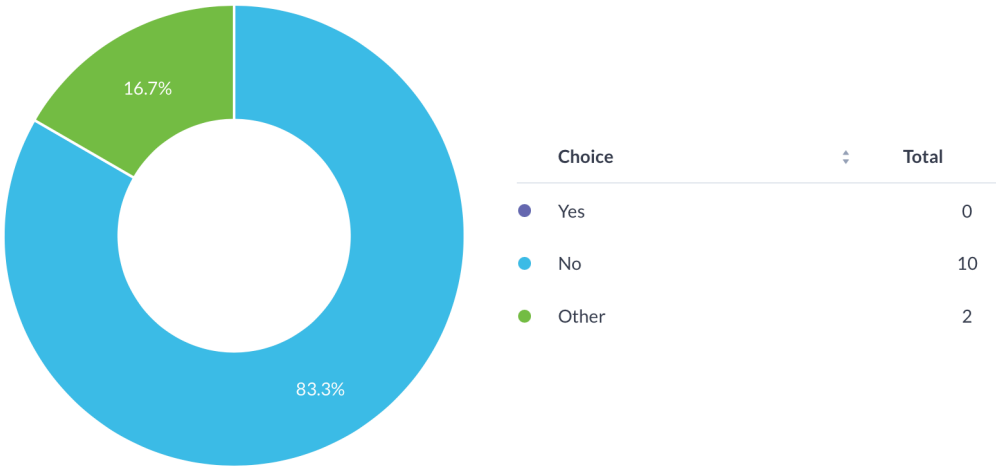
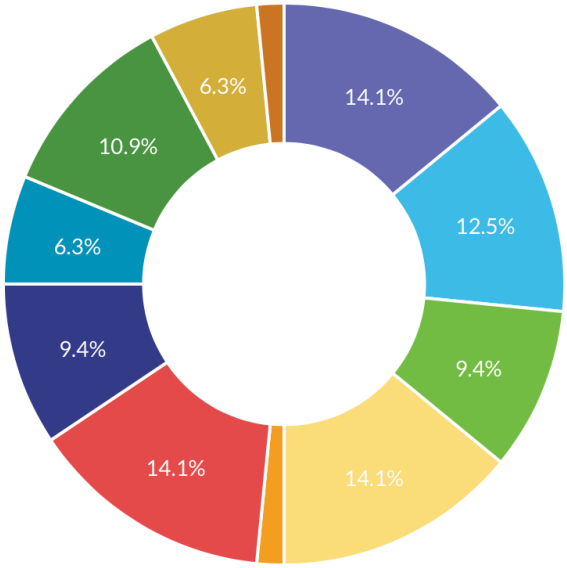


Figure 6.13. Does the lab randomly test for contamination of samples left in long term storage? Multiple Choice.

There were a series of three questions on the questionnaire that pertained to the delivery of evidence to the facilities. Evidence arrives to these facilities in a wide array of methods; the top three methods were UPS, dropped off, and overnighted at 14.1% (Figure 6.6). Once delivered, evidence is placed into storage within 24 hours of being received by the facilities 69.2% of the time (Figure 6.7). Two swabs are the average number of swabs used to collect evidence, which was reported by 66.7% of the facilities.

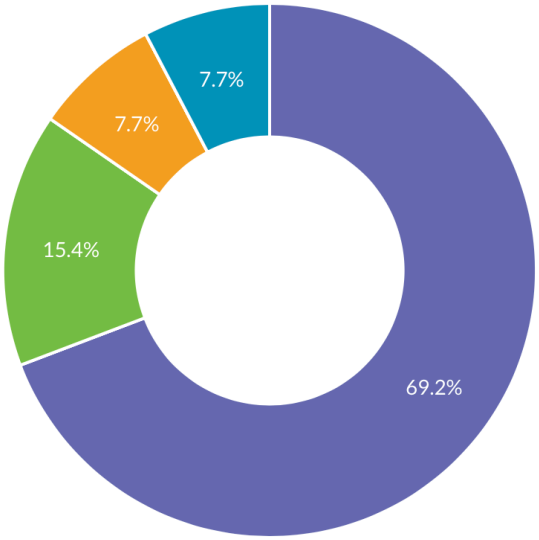
There were a series of eight questions on the questionnaire that pertained to storage of evidence at the facilities. Prior to extractions, genetic evidence was stored at room temperature by 38.9% of facilities, refrigeration by 27.8% of facilities, and either freezer or controlled room temperature 16.7% of facilities. Evidence lockers were used to store dried evidence before and after extractions by 58.3% of facilities. While 41.6% of facilities used open shelving. The average time evidence is stored prior to extractions was 17.92 weeks, with 2 weeks being the lowest time in storage and 51 weeks being the highest. However, 6 weeks was the most frequent length of time reported. Genetic evidence was reported to be stored with multiple samples in one box by 75% of the facilities and in proximity to non-related cases by 58.3% of the facilities (Figure 6.8-6.9). Extracted evidence was reported to be stored in plastic tubes by 41.6% of

facilities and in small evidence envelopes by 33.3% of facilities. The average time extracted evidence is placed back into storage prior to analysis was 3.56 days, with 14 days being the longest time between extraction and analysis.



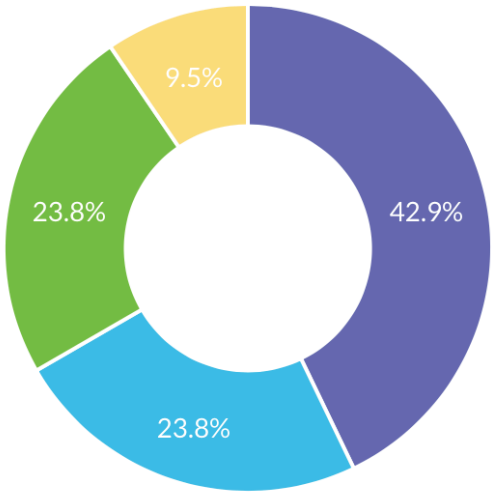
Choice	Total
UPS	9
FedEx	8
USPS	6
Dropped off	9
Picked up by the lab	1
Overnight	9
Express	6
First-class	4
Priority	7
Standard	4
Other	1

Figure 6.14. How is biological evidence mailed to the lab? Select all that apply. Multiple Choice.



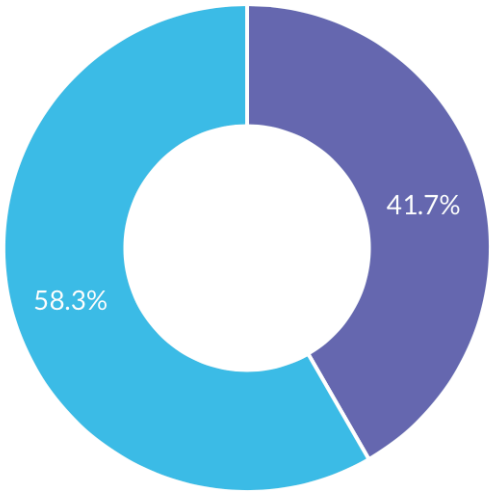
Choice	Total
Less than 24 hours	9
Less than 72 hours	0
A week	2
2-3 weeks	0
A month	1
2-3 months	0
6 months	0
Other	1

Figure 6.15. How long does it take biological evidence from being delivered to the lab to being placed into storage prior to extraction (from point A to point B)? Multiple Choice.



Choice	Total
Multiple samples stored together in a box	9
Multiple samples stored together in an envelope	5
Storage containers physically touching one another	5
Other	2

Figure 6.16. What is the approximate distance between genetic evidence samples? Select all that apply. Multiple Choice.



Choice	Total
Yes, same case	5
Yes, non related case	7
No	0

Figure 6.17. Are genetic evidence samples stored in proximity to other materials or other samples? If yes, is the genetic evidence stored in proximity to other evidence from the same case or non related case? Multiple Choice.

The results of this study consists of two types of analyses: qubit and qPCR. The following sections discuss the results found from performing those tests.

Qubit Results

Temperature

The Qubit was able to detect double stranded DNA in all of the known samples ranging from 0.780 ng/μL to 2.19 ng/μL (Table 6.1-6.3). There was no doubled stranded DNA detected in the refrigerated and frozen blank samples. The Qubit did detect DNA in two freezer controls and one room temperature extraction control. In the blank samples, DNA was only detected in one room temperature sample. The analysis provides an expected range (0.1 to 120 ng) and quality of the double stranded DNA present in the sample (Nakayama et al., 2016).

Humidity

The Qubit was able to detect double stranded DNA in all of the known samples ranging from 0.122 ng/μL to 3.31 ng/μL (Table 6.4-6.6). There was no doubled stranded DNA detected in any of the blank samples. The Qubit did detect DNA in one zero percent humidity extraction control that was extracted alongside the known samples of this variable.

Table 6.5. Room Temperature. Samples beginning with 'B' are the extraction controls, which are listed below its extraction group.

Sample Number	Quantifiable DNA	Sample Number	Quantifiable DNA
1 Known	1.66 ng/μL	**6**	Out of Range
2 Known	0.972 ng/μL	**7**	Out of Range
3 Known	1.91 ng/μL	**8**	Out of Range
4 Known	1.91 ng/μL	**9**	Out of Range
5 Known	1.62 ng/μL	**10**	Out of Range
B4	Out of Range	**B1**	0.0228 ng/μL
11 Blank	0.166 ng/μL	**16**	Out of Range
12 Blank	Out of Range	**17**	Out of Range
13 Blank	Out of Range	**18**	Out of Range
14 Blank	Out of Range	**19**	Out of Range
15 Blank	Out of Range	**20**	Out of Range
B3	Out of Range	**B2**	Out of Range

Table 6.6. Refrigeration. Samples beginning with 'B' are the extraction controls, which are listed below its extraction group.

Sample Number	Quantifiable DNA	Sample Number	Quantifiable DNA
306 Known	1.14 ng/μL	**301**	Out of Range
307 Known	2.19 ng/μL	**302**	Out of Range
308 Known	1.47 ng/μL	**303**	Out of Range
309 Known	1.35 ng/μL	**304**	Out of Range
310 Known	0.780 ng/μL	**305**	Out of Range
B16	Out of Range	**B7**	Out of Range
316 Blank	Out of Range	**311**	Out of Range
317 Blank	Out of Range	**312**	Out of Range
318 Blank	Out of Range	**313**	Out of Range
319 Blank	Out of Range	**314**	Out of Range
320 Blank	Out of Range	**315**	Out of Range
B15	Out of Range	**B8**	Out of Range

Table 6.7. Freezer. Samples beginning with 'B' are the extraction controls, which are listed below its extraction group.

Sample Number	Quantifiable DNA	Sample Number	Quantifiable DNA
506 Known	1.66 ng/μL	**501**	Out of Range
507 Known	0.972 ng/μL	**502**	0.0356 ng/μL
508 Known	1.91 ng/μL	**503**	Out of Range
509 Known	1.91 ng/μL	**504**	Out of Range
510 Known	1.62 ng/μL	**505**	0.0620 ng/μL
B18	Out of Range	**B13**	Out of Range
516 Blank	Out of Range	**511**	Out of Range
517 Blank	Out of Range	**512**	Out of Range
518 Blank	Out of Range	**513**	Out of Range
519 Blank	Out of Range	**514**	Out of Range
520 Blank	Out of Range	**515**	Out of Range
B17	Out of Range	**B14**	Out of Range

Table 6.8. Zero percent relative humidity. Samples beginning with 'B' are the extraction controls, which are listed below its extraction group.

Sample Number	Quantifiable DNA	Sample Number	Quantifiable DNA
101 Known	1.82 ng/μL	**106**	Out of Range
102 Known	2.18 ng/μL	**107**	Out of Range
103 Known	1.52 ng/μL	**108**	Out of Range
104 Known	1.78 ng/μL	**109**	Out of Range
105 Known	0.215 ng/μL	**110**	Out of Range
B6	0.122 ng/μL	**B9**	Out of Range
111 Blank	Out of Range	**116**	Out of Range
112 Blank	Out of Range	**117**	Out of Range
113 Blank	Out of Range	**118**	Out of Range
114 Blank	Out of Range	**119**	Out of Range
115 Blank	Out of Range	**120**	Out of Range
B5	Out of Range	**B10**	Out of Range

Table 6.9. 35% relative humidity. Samples beginning with 'B' are the extraction controls, which are listed below its extraction group.

Sample Number	Quantifiable DNA	Sample Number	Quantifiable DNA
201 Known	0.150 ng/μL	**206**	Out of Range
202 Known	3.31 ng/μL	**207**	Out of Range
203 Known	2.01 ng/μL	**208**	Out of Range
204 Known	0.676 ng/μL	**209**	Out of Range
205 Known	1.28 ng/μL	**210**	Out of Range
B12	Out of Range	**B21**	Out of Range
211 Blank	Out of Range	**216**	Out of Range
212 Blank	Out of Range	**217**	Out of Range
213 Blank	Out of Range	**218**	Out of Range
214 Blank	Out of Range	**219**	Out of Range
215 Blank	Out of Range	**220**	Out of Range
B11	Out of Range	**B22**	Out of Range

Table 6.10. 55% relative humidity. Samples beginning with 'B' are the extraction controls, which are listed below its extraction group.

Sample Number	Quantifiable DNA	Sample Number	Quantifiable DNA
401 Known	0.186 ng/μL	**406**	Out of Range
402 Known	0.123 ng/μL	**407**	Out of Range
403 Known	0.208 ng/μL	**408**	Out of Range
404 Known	2.64 ng/μL	**409**	Out of Range
405 Known	4.56 ng/μL	**410**	Out of Range
B24	Out of Range	**B19**	Out of Range
411 Blank	Out of Range	**416**	Out of Range
412 Blank	Out of Range	**417**	Out of Range
413 Blank	Out of Range	**418**	Out of Range
414 Blank	Out of Range	**419**	Out of Range
415 Blank	Out of Range	**420**	Out of Range
B23	Out of Range	**B20**	Out of Range

qPCR

The Plexor® analysis, which was used to interpret the qPCR results from the Mx3000P®, provided insight into the samples and allowed for quantifying different types of DNA potentially present. After establishing the standard curve for the assigned dyes FAM (autosomal DNA) and CO560 (Y-chromosomal DNA), the concentration is calculated based on the DNA type. The quantitation cycle, during which the DNA is detected, is reported by the Cq. If the melt threshold is in the expected target melt temperature range (81-83˚C for CO565; 79-81˚C for FAM or CR610), then the Tm? will indicate with a 'Yes', 'No', or 'No Call'. A 'Yes' result indicates the amplification is within the expected melt temperature range and has crossed the melt threshold. While a 'No' result indicates the amplification is not within the expected melt temperature range. A 'No Call' result indicates the amplification is within the expected melt temperature range, but the amplification does not cross the melt threshold (Plexor® HY System Manual). The melt threshold is determined by a percentage in which the denaturing double stranded DNA amplicon

signal must surpass the expected change in fluorescence over change in temperature. The melt curve is influenced by the number of amplicons in the sample (Steffen, 2016).

Temperature

The Plexor® analysis was conducted on the known samples for the temperature variable. Autosomal DNA was detected in all the known samples, which the concentrations ranged from 5.696 E-01 to 9.354 ng/μL. Y-chromosomal DNA was detected in all the known samples, which the concentrations ranged from 2.162 E0 to 6.527 E-01ng/μL (Appendix D).

The Plexor® analysis was conducted on the blank samples for the temperature variable. Autosomal DNA was detected in some of the blank samples (Appendix D). In the room temperature samples, swabs 11 and 13 detected Autosomal DNA. In the refrigerated samples, swabs 316, 318, and 320 detected autosomal DNA. In the freezer samples, swabs 517, 518, 519, and 520 detected autosomal DNA. However, these samples did not detect Y-chromosomal DNA. Y-chromosomal DNA was only detected in swab 15 at a concentration of 6.19E-04 ng/μL, which was a room temperature sample (Table 6.7). However, the amplification is not within the expected target melt temperature when the threshold is at 25%. When the threshold was reduced to 5%, sample 15 changed to a 'No Call,' demonstrating that the sample carried too little DNA to amplify past the melt threshold.

Table 6.11. CO560-Y qPCR results of the blank temperature samples. Samples that cross the amplification threshold have a Cq listed, which indicates the quantitation cycle. The concentration is of the Y-chromosomal DNA. Tm? indicates if the melt threshold is in the expected target melt temperature range. Two thresholds were analyzed 25% and 5%.

Sample Number	Sample Type	Temperature	Cq	Concentration	Tm? 25%	Tm? 5%
11	Blank	Room	N/A	N/A	NO	NO
12	Blank	Room	N/A	N/A	NO	NO
13	Blank	Room	N/A	N/A	NO	NO
14	Blank	Room	N/A	N/A	NO	NO
15	Blank	Room	36.36	6.19E-04	NO	NO CALL
316	Blank	Refrigeration	N/A	N/A	NO	NO
317	Blank	Refrigeration	N/A	N/A	NO	NO
318	Blank	Refrigeration	N/A	N/A	NO	NO
319	Blank	Refrigeration	N/A	N/A	NO	NO
320	Blank	Refrigeration	N/A	N/A	NO	NO
516	Blank	Freezer	N/A	N/A	NO	NO
517	Blank	Freezer	N/A	N/A	NO	NO
518	Blank	Freezer	N/A	N/A	NO	NO
519	Blank	Freezer	N/A	N/A	NO	NO
520	Blank	Freezer	N/A	N/A	NO	NO

The Plexor® analysis was conducted on the control samples for the temperature variable. An initial test was conducted of the control samples. Based on the qPCR results of controls, the entire plate of samples had been contaminated at some point during the qPCR preparations. Therefore, another plate of the control samples needed to be conducted to determine if contamination occurred during the storage process. However, because of the limited number solution left in the Plexor® HY System kit only 4 of the 10 control swabs of each temperature variable could be tested. Autosomal DNA was detected in some of the control samples (Appendix D). During the first qPCR run of the controls, all the samples detected autosomal DNA. Except for swabs 511 and 502, which are freezer samples, Y-chromosomal DNA was also detected during this run. In the room temperature control samples, swabs 6 and 18 detected autosomal DNA. In the freezer control samples, swab 511 detected autosomal DNA. The extraction swab for the blank refrigerator samples, B15, detected autosomal DNA. Y-

chromosomal was detected in some of the samples. In the room temperature controls, swabs 6 and 20 detected Y-chromosomal DNA ranging from 3.706 E-03 to 2.957 E-02 ng/μL. In the freezer samples, swab 504 detected Y-chromosomal DNA at a concentration of 4.597 E-03 ng/μL. The extraction swab B15 also detected Y-chromosomal DNA at a concentration of 1.311 E-02 ng/μL. However, the amplification is not within the expected target melt temperature when the threshold is at 25%. When the threshold was reduced to 5%, samples 20 and 315 changed to a 'No Call'. Of those samples, 20 was the only sample with a Y-chromosomal DNA concentration (Table 6.8).

Table 6.12. CO560-Y qPCR results of the control temperature samples from the second run. Samples that cross the amplification threshold have a Cq listed, which indicates the quantitation cycle. The concentration is of the Y-chromosomal DNA. Tm? indicates if the melt threshold is in the expected target melt temperature range. Two thresholds were analyzed 25% and 5%.

Sample Number	Sample Type	Temperature	Cq	Concentration	Tm? 25%	Tm? 5%
6	Control	Room	33.04	2.957 E-02	NO	NO
17	Control	Room	N/A	N/A	NO	NO
18	Control	Room	N/A	N/A	NO	NO
20	Control	Room	33.04	3.706 E-03	NO	NO CALL
303	Control	Refrigeration	N/A	N/A	NO	NO
304	Control	Refrigeration	N/A	N/A	NO	NO
311	Control	Refrigeration	N/A	N/A	NO	NO
315	Control	Refrigeration	N/A	N/A	NO	NO CALL
501	Control	Freezer	N/A	N/A	NO	NO
504	Control	Freezer	36.52	4.597 E-03	NO	NO
511	Control	Freezer	N/A	N/A	NO	NO
513	Control	Freezer	N/A	N/A	NO	NO
B15	Extraction Control: Blank	Refrigeration	34.56	1.311 E-02	NO	NO

Humidity

The Plexor® analysis was conducted on the known samples for the humidity variable. Autosomal DNA was detected in all the known samples, which the concentrations ranged from

5.352 E-03 to 6.670 ng/μL. Y-chromosomal DNA was detected in all the known samples, which the concentrations ranged from 8.245 E-03 to 2.164 ng/μL (Appendix D).

The Plexor® analysis was conducted on the blank samples for the humidity variable. Autosomal DNA was detected in some of the blank samples, which had not been exposed to DNA (Appendix D). In the approximate zero humidity samples, swabs 114 and 115 detected Autosomal DNA. In the 35% humidity samples, swabs 212, 213, and 214 detected autosomal DNA. In the 55% samples, swabs 412, 413, 414, and 415 detected autosomal DNA. However, the swabs 114, 115, 212, and 413 did not detect Y-chromosomal DNA. Y-chromosomal DNA concentration was detected in at least one swab of each tested humidity level. In the approximate zero humidity samples, swab 111 detected 2.472E-04 ng/μL of Y-chromosomal DNA. In the 35% humidity samples, swab 213 detected 2.896E-04 ng/μL of Y-chromosomal DNA. In the 55% humidity samples, swabs 411, 412, 414, and 415 detected Y-chromosomal DNA that ranged from 3.289E-03 to 3.656E-04 ng/μL. However, the amplification is not within the expected target melt temperature when the threshold is at 25%. When the threshold was reduced to 5%, samples 113, 115, 411, and 413 changed to a 'No Call'. Of those samples, 411 was the only sample with a Y-chromosomal DNA concentration (Table 6.9).

Table 6.13. CO560-Y qPCR results of the blank humidity samples. Samples that cross the amplification threshold have a Cq listed, which indicates the quantitation cycle. The concentration is of the Y-chromosomal DNA. Tm? indicates if the melt threshold is in the expected target melt temperature range. Two thresholds were analyzed 25% and 5%.

Sample Number	Sample Type	Humidity	Cq	Concentration	Tm? 25%	Tm? 5%
111	Blank	~0	37.82	2.472E-04	NO	NO
112	Blank	~0	N/A	N/A	NO	NO
113	Blank	~0	N/A	N/A	NO	NO CALL
114	Blank	~0	N/A	N/A	NO	NO
115	Blank	~0	N/A	N/A	NO	NO CALL
211	Blank	35	N/A	N/A	NO	NO
212	Blank	35	N/A	N/A	NO	NO
213	Blank	35	37.57	2.896E-04	NO	NO
214	Blank	35	N/A	N/A	NO	NO
215	Blank	35	N/A	N/A	NO	NO
411	Blank	55	37	3.998E-04	NO	NO CALL
412	Blank	55	33.59	3.289E-03	NO	NO
413	Blank	55	N/A	N/A	NO	NO CALL
414	Blank	55	33.8	2.893E-03	NO	NO
415	Blank	55	37.13	3.656E-04	NO	NO

The Plexor® analysis was conducted on the control samples for the humidity variable. An initial test was conducted of the control samples. Based on the qPCR results of controls, the entire plate of samples had been contaminated at some point during the qPCR preparations. Therefore, another plate of the control samples needed to be conducted to determine if contamination occurred during the storage process. However, because of the limited number solution left in the Plexor® HY System kit only 4 of the 10 control swabs of each humidity variable could be tested. Autosomal DNA was detected in some of the control samples (Appendix D). In the 35% humidity samples, swabs 206, 207, 217, and 218 detected autosomal DNA at a concentration ranging from 7.128 E-04 to 1.123 E-02 ng/μL. Swabs 206, 207, and 218 also detected Y-chromosomal DNA ranging from 4.506 E-03 to 1.927 E-03 ng/μL. There was no detection of autosomal of Y-chromosomal DNA for the 35% humidity extraction control

samples. In the 55% humidity extraction controls, autosomal DNA was detected in swab B24 at a concentration of 5.816 E-04 ng/μL. During the first qPCR run of the controls, all the samples detected autosomal DNA. Except for swabs 118, 408, B10, and B19 detected Y-chromosomal DNA during this run. However, the amplification is not within the expected target melt temperature when the threshold is at 25%. When the threshold was reduced to 5%, samples 410 and 418 changed to a 'No Call' and 409 changed to a 'Yes', which none of the samples indicated a Y-chromosomal DNA concentration (Table 6.10).

Table 6.14. CO560-Y qPCR results of the control humidity samples from the second run. Samples that cross the amplification threshold have a Cq listed, which indicates the quantitation cycle. The concentration is of the Y-chromosomal DNA. Tm? indicates if the melt threshold is in the expected target melt temperature range. Two thresholds were analyzed 25% and 5%.

Sample Number	Sample Type	Humidity	Cq	Concentration	Tm? 25%	Tm? 5%
106	Control	~0	37.07	3.419 E-03	NO	NO
108	Control	~0	37.65	2.506 E-03	NO	NO
119	Control	~0	37.93	2.163 E-03	NO	NO
120	Control	~0	N/A	N/A	NO	NO
409	Control	55	N/A	N/A	NO	YES
410	Control	55	N/A	N/A	NO	NO CALL
416	Control	55	N/A	N/A	NO	NO
418	Control	55	N/A	N/A	NO	NO CALL
B5	Extraction Control: Blank	~0	N/A	N/A	NO	YES
B10	Extraction Control: Control	~0	N/A	N/A	NO	NO
206	Control	35	35.32	7.866 E-03	NO	NO
207	Control	35	34.20	2.603 E-03	NO	NO
218	Control	35	35.57	1.768 E-02	NO CALL	NO
220	Control	35	N/A	N/A	NO	NO

STR

STR analysis was attempted using the PowerPlex® Fusion System kit, which were then ran through GeneMapper version 3.6. However, results came back inconclusive and could not generate an STR profile for neither standards nor the known samples.

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Chapter 7: Discussion & Conclusion

This chapter discusses the observations, findings, and readdresses the hypotheses of this research on the relationship temperature and humidity has with contamination in storage. If contamination during storage is possible, the findings suggest the DNA concentration is too low to interfere with generating a STR profile. The following chapter addresses the observations that led to this conclusion on storage contamination.

Qubit

The Qubit analysis provides an expected range (0.1 to 120 ng) to quantify the double-stranded DNA in the sample (Nakayama et al., 2016). Single sample analysis can produce varying results, as seen in some of the control samples of this study, which resulted in both quantifiable DNA and 'out of range' for a single sample. Therefore, the results from a Qubit analysis should not influence the interpretation of additional analyses. Nakayama et al. (2016) found Qubit analysis accuracy to be dependent on the initial condition of the DNA sample source. Compared to two other quantification methods, NanoDrop and qPCR, the Qubit analysis was not consistently the most accurate. For instance, the Qubit quantification did not correspond to the dilution ratio in the samples where the DNA had been extracted from frozen tissue cells. Thus, the Qubit indicated lower values of double-stranded DNA (Nakayama et al., 2016). Because the present study delt with an initial low DNA concentration, any contamination or stochasticity in pipetting in the blank samples could potentially be missed with the Qubit analysis.

The quantifiable DNA detected by the qubit in the few controls and extraction controls could indicate two scenarios. Two controls detected quantifiable DNA and two extraction controls (one from a known extraction group and one from a control extraction group). First, the

swabs used in the study potentially were contaminated during the manufacturing process, therefore, were not DNA-free (despite being advertised as such). Second, the samples were contaminated during the extraction process potentially caused by the lab bench or equipment not being wiped down efficiently enough. Therefore, if a high concentration of DNA is present in the blank or control swab results, then the contamination is more plausibly from an external source other than storage.

Nonspecific Amplification

The presence of nonspecific amplification within the samples was indicated by the qPCR and STR results. First, many of the qPCR blank and control swabs either detected a DNA concentration without making a call, or did not determine a DNA concentration with made call. Second, the attempted STR analysis was unable to provide results for the known swab samples. But why does the presence of nonspecific amplification matter? Nonspecific amplification can be primer-dimers, mis-priming, or inhibitors, which interfere with the results as the inhibitors will bind to the DNA and change the shape of the melt curve. If the melt curve is outside the expected melt temperature range, this indicates nonspecific amplification to be present in the samples (Krenke et al., 2008; Thompson, 2010; Thompson et al., 2014). Therefore, it is crucial to maintain the integrity of the DNA, as the analysis kit and DNA concentration influence the downstream detection of a STR profile. The Plexor® HY kit has trouble detecting profiles from DNA concentrations beginning between 0.62 pg/μL to 0.21 pg/μL (Krenke et al., 2008; Ginart et al., 2019). If nonspecific amplification does not interfere with the samples, then any potential contamination from storage is too low to detect within a STR profile. Therefore, any contamination that is detected is a result from another part of the forensic investigation process,

such as evidence collection or DNA extraction. This data therefore reaffirms the confidence level in the STR profiles discovered from genetic evidence.

Temperature, Storage, and Contamination

Based on the analyses, temperature does not influence contamination during storage. Neither refrigeration nor the freezer detected Y-chromosomal DNA on the swabs in close proximity to those that had been exposed to DNA. However, DNA concentration was detected in a freezer control and a refrigeration extraction control. Yet, the Plexor® analysis did not call amplification within the expected melt temperature range at both 25% and 5% melt threshold, which indicates the detected DNA concentration is a result of primer dimer or nonspecific amplification.

The effect room temperature has on contamination during storage needs further investigation. During the Qubit analysis, a room temperature blank swab (Blank 11) detected 0.166 ng/μL quantifiable double-stranded DNA; however, in the qPCR analysis this sample did not detect a Y-chromosomal DNA concentration, but autosomal DNA concentration was detected. Therefore, it is possible the DNA present in swab 11 is a result of a different source of contamination. During qPCR analysis, another room temperature blank swab (Blank 15) detected a Y-chromosomal DNA concentration. At a 25% melt threshold, there was no amplification detected within the melt temperature range for swab 15. When the melt threshold was reduced to 5%, amplification was detected within the melt temperature range but did not cross the melt threshold for swab 15. There is likely nonspecific amplification interfering with this sample as STR analysis could not provide results or identification of the DNA source. If Y-chromosomal DNA was present in swab 15, which would suggest storage contamination at room temperature, then it cannot be parsed further because of the amplification interference.

However, two control swabs detected a Y-chromosomal DNA concentration. Yet, the Plexor® analysis did not call amplification within the expected melt temperature range, which indicates the detected DNA concentration is a result of primer dimer or nonspecific amplification. Control swab 20, resulted in a 'No Call' (amplification detected within the temperature range, but does not pass the melt threshold) when the melt threshold was at 5%. Therefore, it is possible that contamination occurred during qPCR setup or through the kit itself, as the high concentration of the standard DNA included with the Plexor® is located within the same, small box as the other solution materials.

Humidity, Storage, and Contamination

Based on the analyses, humidity potentially influences contamination during storage. Neither zero percent humidity nor 35% humidity detected Y-chromosomal DNA at a 25% melt threshold. Zero percent humidity blank swabs 113 and 115 did result in a 'No Call' when the melt threshold was at 5%, however, these swabs did not detect a DNA concentration, so primer dimer is likely present in these samples. A DNA concentration was detected in swabs 111 and 213, but there was no amplification detected within the melt temperature range, suggesting the concentration is a result of nonspecific amplification.

The effect 55% humidity has on contamination during storage needs further investigation. Four of the five blank swabs detected a concentration of Y-chromosomal DNA. However, there was no amplification detected within the melt temperature range at a 25% melt threshold. At a 5% melt threshold, there was amplification detected within the melt temperature range but did not cross the melt threshold for swab 411 and 413s. There is likely nonspecific amplification interfering with this sample as STR analysis could not provide results. If Y-chromosomal DNA was present in swab 411 and 413, which would suggest storage contamination when humidity is

above 55%, then it cannot be parsed further because of the amplification interference. Despite this, the increased detection of a Y-chromosomal concentration in the 55% humidity swabs reflects the effects humidity has on DNA beginning at 50% relative humidity. If high levels of humidity causes contamination, then the effects of rehydration and denaturation of DNA should be analyzed further (Bonnet et al., 2009; Colotte et al., 2011; Tan et al., 2021).

The qPCR results for the humidity controls indicates interference. Three of the 0% and 35% humidity controls swabs detected a DNA concentration, which were not amplified within the melt temperature range. A 55% humidity control swab and a 0% extraction control detected amplification above a 5% melt threshold despite there being no DNA concentration detected. Another two 55% humidity control swabs detected amplification within the melt temperature range that did not cross the 5% melt threshold, despite there being no DNA concentration detected. These detections of amplification are likely an indication of primer dimer, which is a PCR by-product (Dash et al., 2020).

Hypotheses

After addressing my findings, we need to readdress the original hypotheses set out by this research, in chapter 5, to consider how the results should be assessed. Based on this research the hypotheses must be accepted until more in-depth research can be conducted.

Hypothesis I: The likelihood of DNA migration through a material is not influenced by an increase in temperature.

The hypothesis is potentially disproven based on the results. If hypothesis I were true, then contamination would be present at all temperatures or absent altogether. The results indicate contamination during the storage method used does not occur when the temperature is below 8˚C. However, the qPCR result for one room temperature blank swab detected DNA

amplification within the melt temperature range, but the amplification did not cross the melt threshold, thus resulting in a 'No Call' on the DNA concentration in the sample. Therefore, temperatures above 8 ˚C potentially influences the movement of DNA.

Hypothesis II: At a given temperature, neither vapor pressure nor humidity influences the movement of DNA.

The hypothesis is potentially partially disproven based on the results. If hypothesis II were true, then contamination would be consistently present at different humidity levels or absent altogether. The results indicate humidity does not influence the movement of DNA below 35% humidity. However, the qPCR results for one 55% humidity blank swab detected DNA amplification within the melt temperature range, but the amplification did not cross the melt threshold, thus resulting in a 'No Call' on the DNA concentration in the sample. Therefore, high humidity potentially influences the movement of DNA.

Limitations

With forensic evidence, there is variation in how the evidence is stored, collected, and packaged. Because of this, the results of the research would need to be tested with different collection material and packaging to determine if similar results occur under different conditions.

Sample size is another limitation. Each variable tested for contamination of five blank swabs amongst five known swabs. If contamination were to occur in the variables, whose samples did not detect contamination, then the possibility of contamination potentially happens at a lower rate.

Implications

Despite being unable to determine if temperature or humidity influence storage contamination, there are three implications of the research. First, contamination does not occur

during these storage conditions when the temperature is below 8˚C. This could be a result of a restriction in movement of DNA based on the temperature, which has been observed in leaching studies (Hebsgaard et al., 2009; Arnold et al., 2011; Andersen et al., 2012). In addition, less water vapor is needed to reach 100% relative humidity as temperature decreases, therefore, there is more water vapor present in storage at room temperature than in storage below 8˚C. If forensic facilities can access either refrigerators or freezers, then those should be preferred storage method for buccal swabs. Using refrigeration or freezers over room temperature will add another layer to maintaining evidence integrity.

Second, room temperature potentially has an influence on contamination during storage. This is inferred by the humidity results detecting a DNA concentration in at least one sample for each humidity level tested along with one of the room temperature swabs. Therefore, further research is needed to analyze the effects room temperature has on DNA in relation to contamination. Since room temperature is not a specifically designated range within forensic lab manuals, all temperatures above 8˚C should be tested.

Lastly, high levels of humidity potentially have an influence on contamination during storage. As discussed above, humidity begins to have an effect on DNA beginning at 50% relative humidity (Bonnet et al., 2009; Colotte et al., 2011; Tan et al., 2021). However, room temperature storage conditions do not control for humidity, nor temperature-controlled storage conditions which allow humidity up to 60% (Ballou et al., 2013). If room temperature storage must be used, then the maximum humidity levels need to be reduced to below 50% humidity. Further research is needed to analyze the effects high levels of humidity has on DNA in relation to contamination. The humidity levels tested should focus two points where DNA begins to rehydrate and the DNA structure changes (Westhof, 1988; Bonnet et al., 2009): from 45% to

50% relative humidity, and 70% to 75% relative humidity at 1% increments. Then between 50% to 70% test at 5% increments. This would help determine if potential contamination is a result of the rehydration of DNA or its structural change and the need to determine alternative storage methods to prevent those changes. If rehydration was the cause of contamination, this would further support the need to ensure genetic evidence is dried prior to packaging.

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Appendix A: Temperature Samples; Red Numbers indicate Samples that were introduced to DNA

Room Temperature Known/Blank Samples

#1			#12									#11			#2
						#13			#3						
	#14			#4							#5			#15	

Room Temperature Control Samples

6			16									17			7
						8			18						
	19			9							10			20	

Refrigeration Known/Blank Samples

306			316									317			307
						308			318						
	319			309							310			320	

Refrigeration Control Samples

301			311									312			302
						303			313						
	304			314							305			315	

Freezer Known/Blank Samples

506			516									517			507
						508			518						
	519			509							510			520	

Freezer Control Samples

501			511									512			502
						503			513						
	514			504							505			515	

Appendix B: Humidity Samples; Red Numbers indicate Samples that were introduced to DNA

~0% Humidity Known/Blank Samples

101			111									112			102
						103			113						
	104			114							105			115	

~0% Humidity Control Samples

106			107									108			109
						303			313						
	117			118							119			120	

36% Humidity Known/Blank Samples

201			211									212			202
						203			213						
	204			214							205			215	

36% Humidity Control Samples

206			216									217			207
						208			218						
	219			209							210			220	

55% Humidity Known/Blank Samples

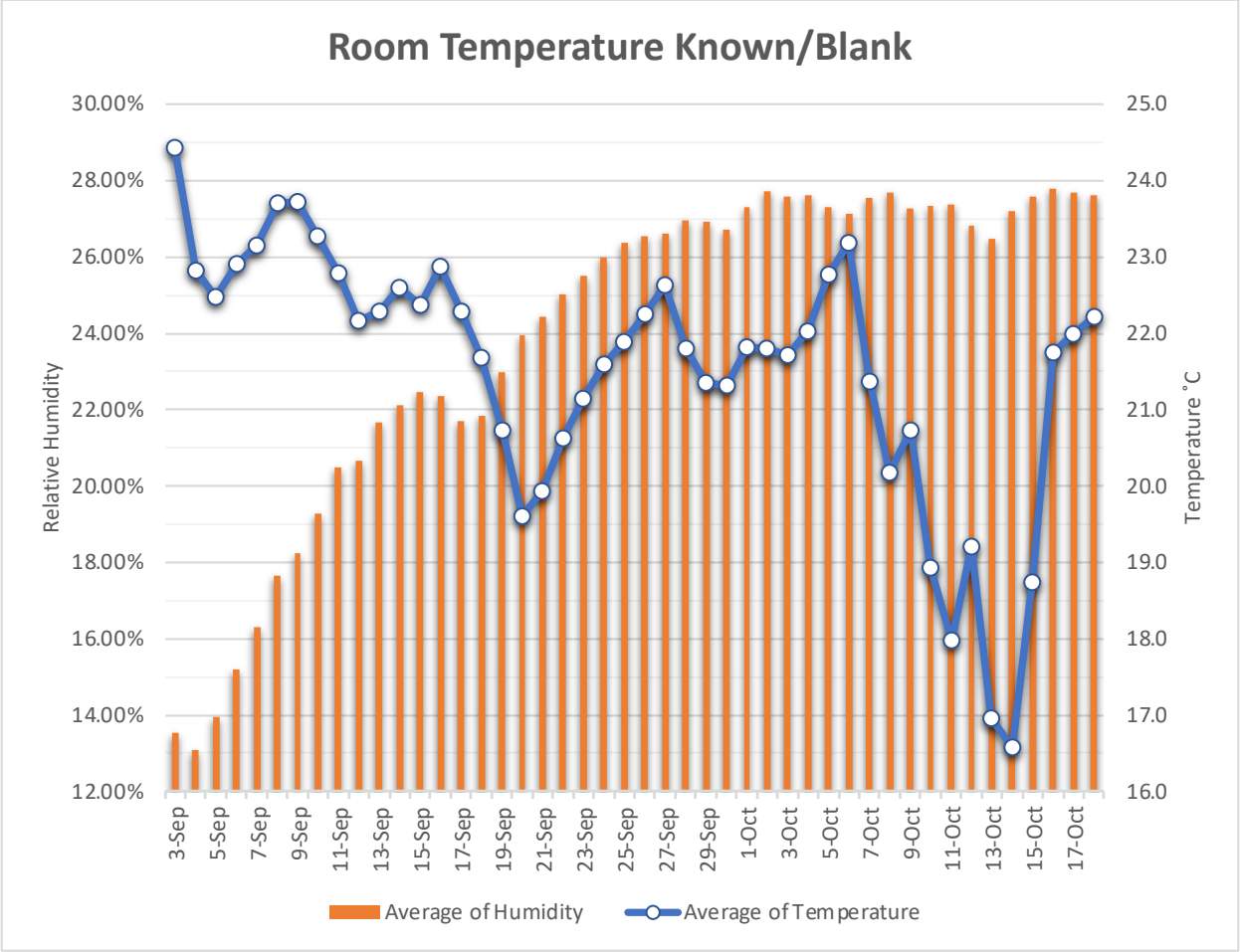
401			411									412			402
						403			413						
	414			404							405			415	

55% Humidity Control Samples

406			416									417			407
						408			418						
	419			409							410			420	

Appendix C: TraceableGo™ Datalogger Hygrometer

Temperature: Room Temperature



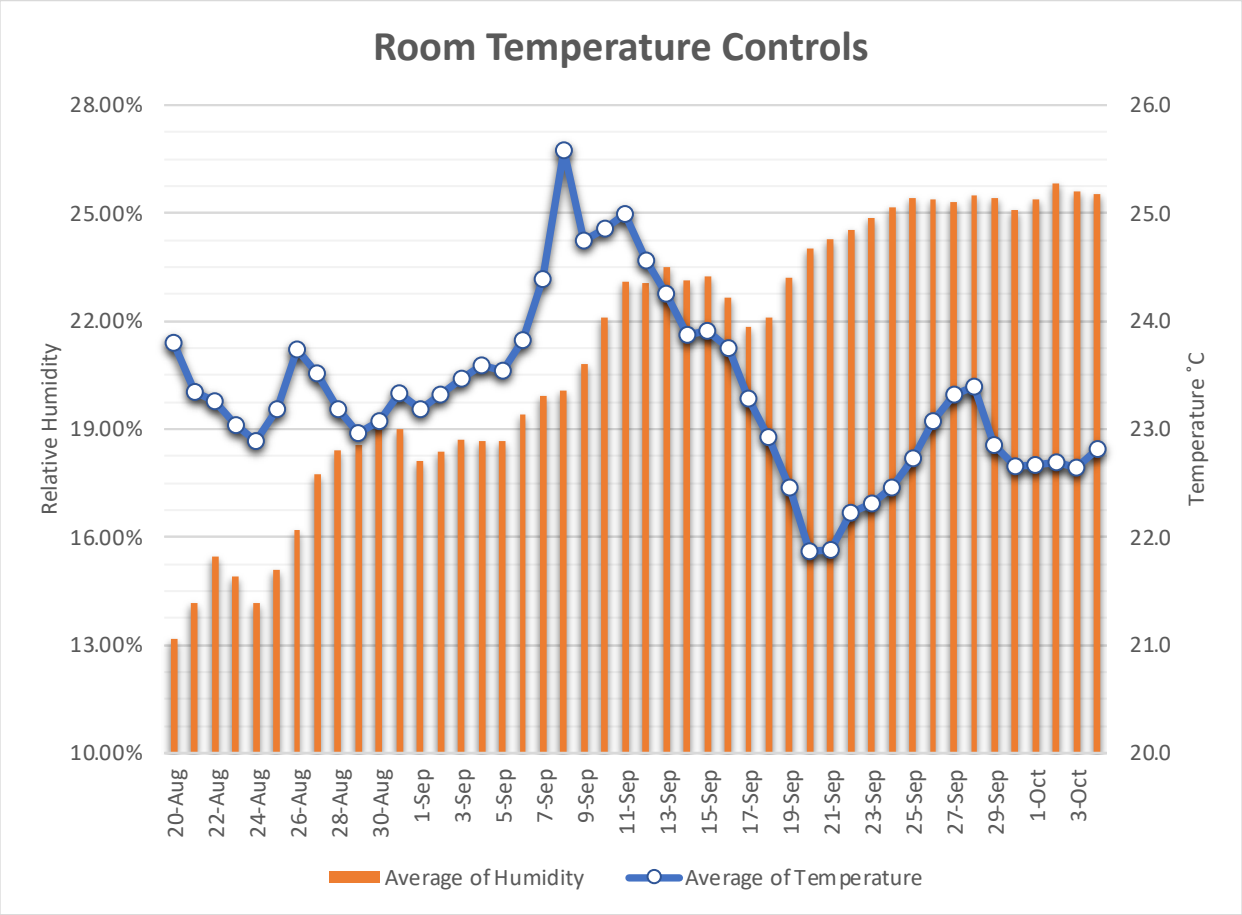
HUMIDITY SUMMARY

Minimum Reading on 09/04/2021 11:09 AM: 12.20%
Maximum Reading on 10/04/2021 02:00 PM: 28.10%
Average Reading: 24.06%

TEMPERATURE SUMMARY

Minimum Reading on 10/14/2021 08:51 AM: 16.20C
Maximum Reading on 10/09/2021 11:56 AM: 36.40C
Average Reading: 21.48C

Temperature: Room Temperature Control



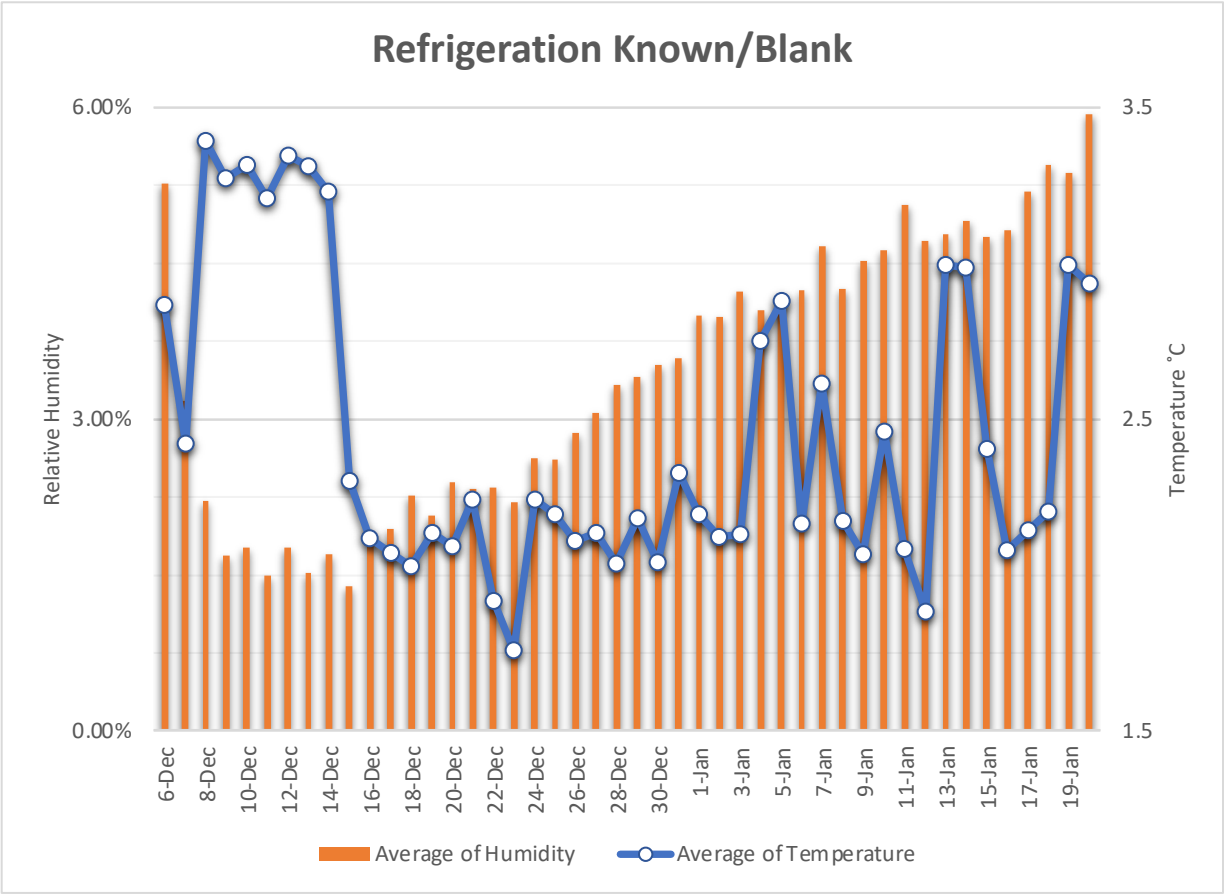
HUMIDITY SUMMARY

Minimum Reading on 08/20/2021 06:14 PM: 12.10%
Maximum Reading on 10/02/2021 04:56 AM: 26.00%
Average Reading: 21.25%

TEMPERATURE SUMMARY

Minimum Reading on 09/20/2021 05:25 PM: 21.60C
Maximum Reading on 09/08/2021 05:42 PM: 25.80C
Average Reading: 23.33C

Temperature: Refrigeration



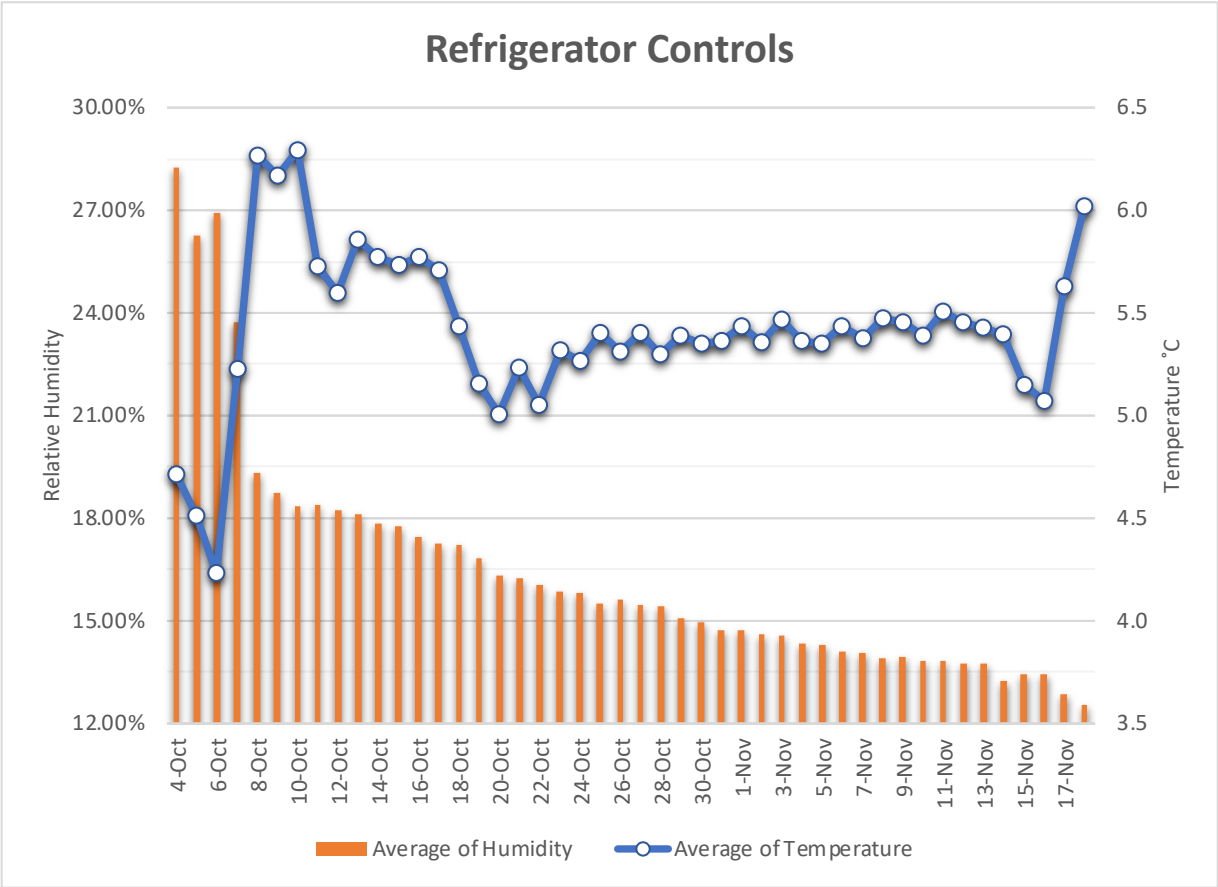
HUMIDITY SUMMARY

Minimum Reading on 12/15/2021 03:43 PM: .80%
Maximum Reading on 01/20/2022 02:52 PM: 18.70%
Average Reading: 3.39%

TEMPERATURE SUMMARY

Minimum Reading on 01/18/2022 03:05 PM: .40C
Maximum Reading on 01/20/2022 02:52 PM: 14.00C
Average Reading: 2.44C

Temperature: Refrigeration Control



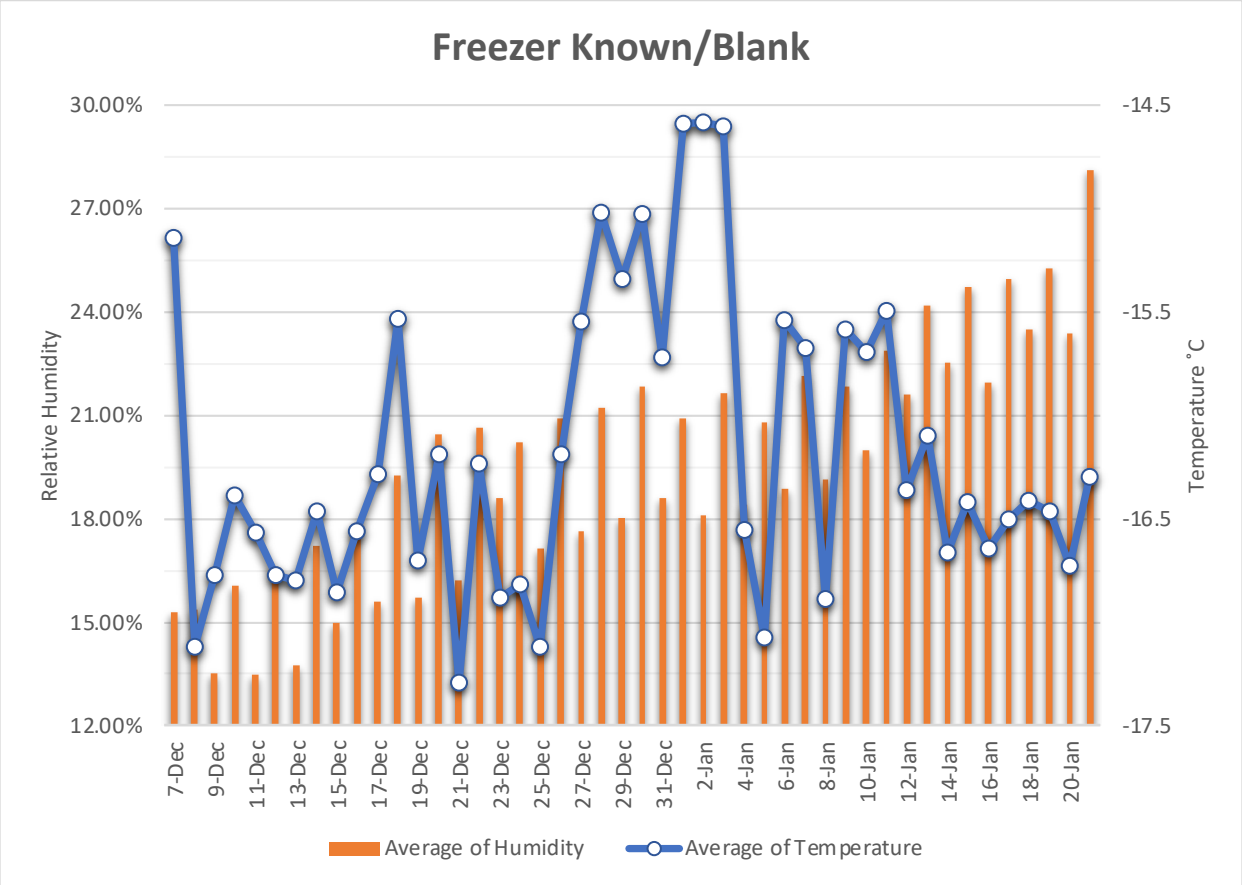
HUMIDITY SUMMARY

Minimum Reading on 11/18/2021 03:47 AM: 11.30%
Maximum Reading on 10/06/2021 06:06 PM: 32.10%
Average Reading: 16.30%

TEMPERATURE SUMMARY

Minimum Reading on 10/11/2021 10:57 AM: 2.60C
Maximum Reading on 10/05/2021 04:41 PM: 8.50C
Average Reading: 5.42C

Temperature: Freezer



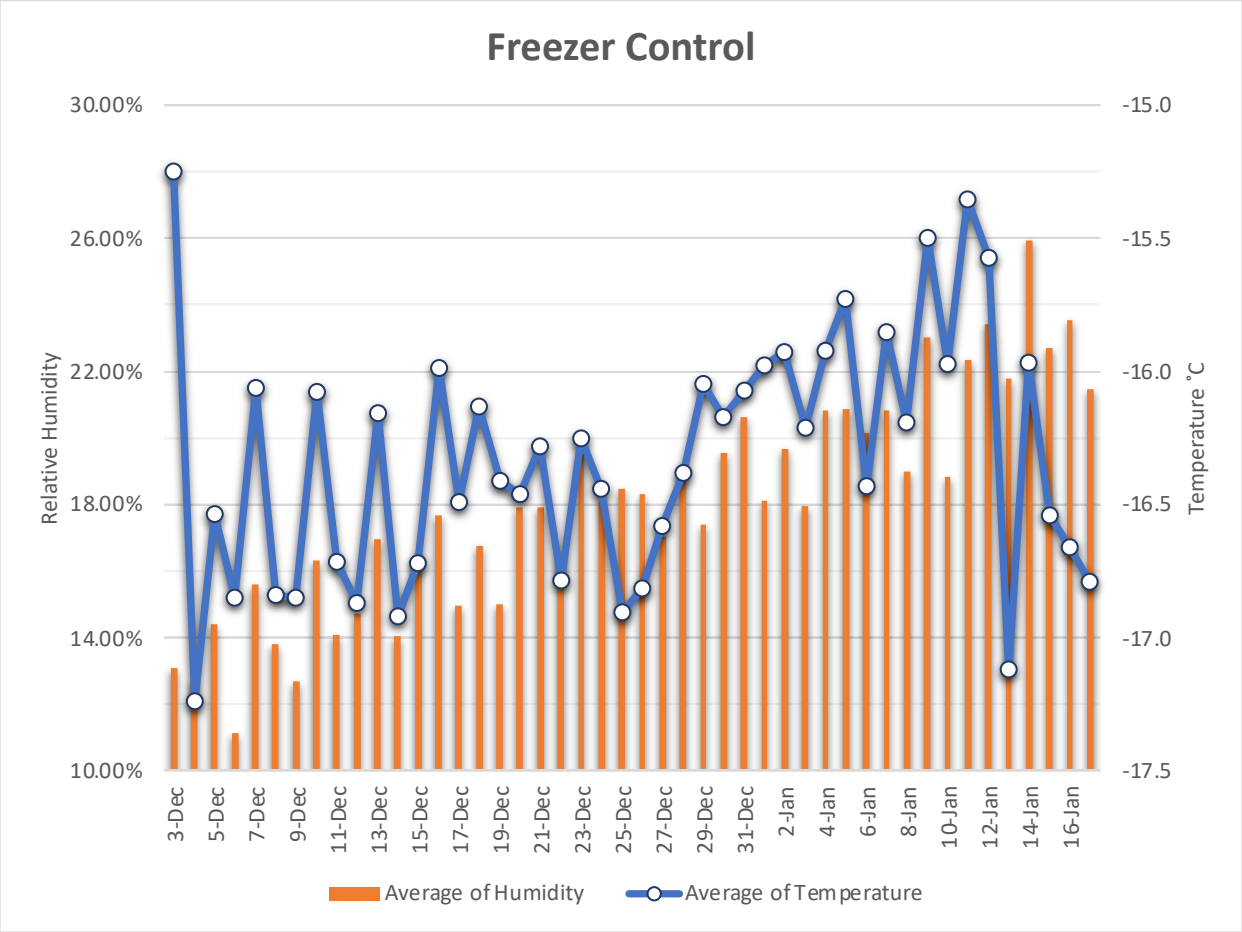
HUMIDITY SUMMARY

Minimum Reading on 12/08/2021 03:14 AM: 11.10%
Maximum Reading on 12/20/2021 03:58 AM: 44.60%
Average Reading: 19.52%

TEMPERATURE SUMMARY

Minimum Reading on 12/24/2021 10:39 AM: -20.00C
Maximum Reading on 01/03/2022 03:28 AM: -1.30C
Average Reading: -16.19C

Temperature: Freezer Control



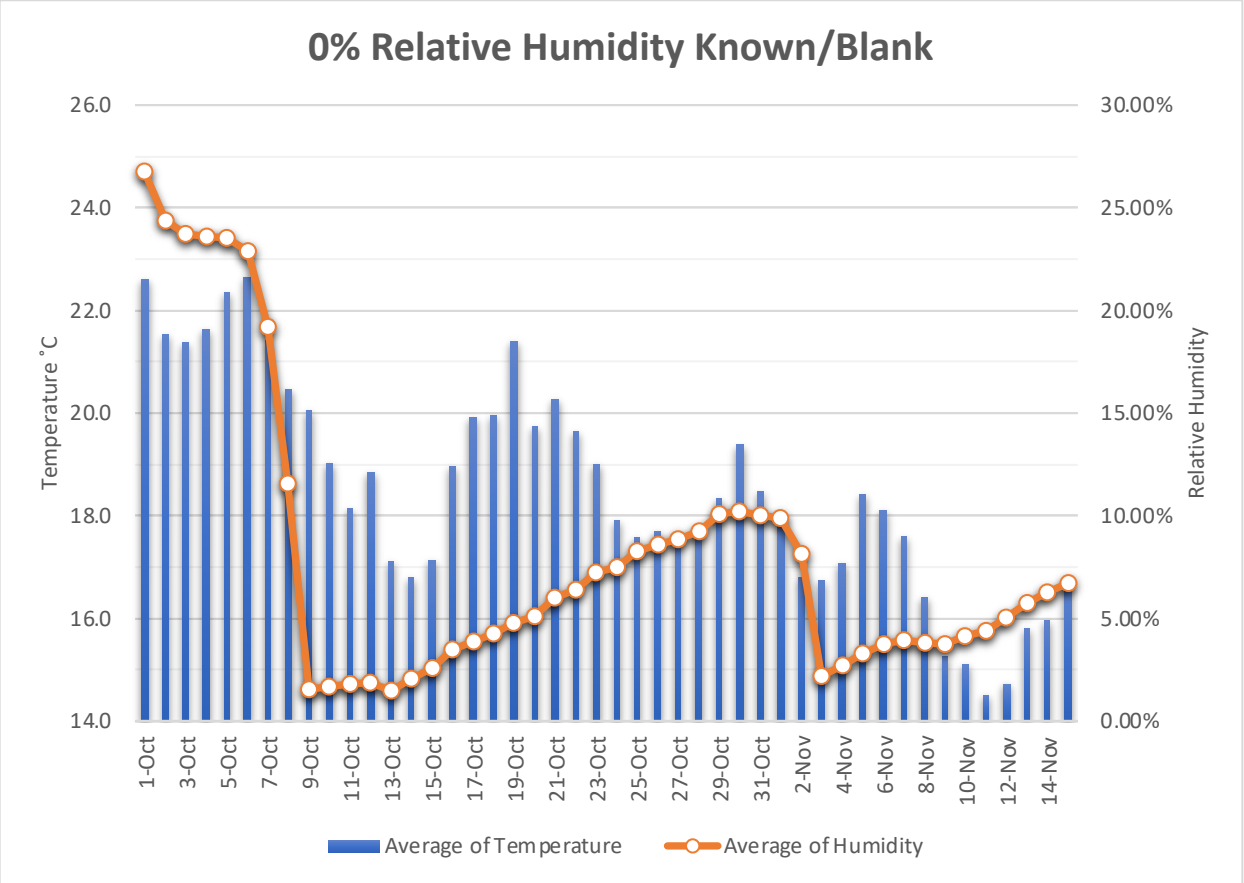
HUMIDITY SUMMARY

Minimum Reading on 12/03/2021 02:29 PM: 8.30%
Maximum Reading on 01/05/2022 10:36 PM: 63.80%
Average Reading: 18.09%

TEMPERATURE SUMMARY

Minimum Reading on 12/08/2021 06:31 PM: -18.90C
Maximum Reading on 01/12/2022 06:17 PM: -2.50C
Average Reading: -16.33C

Humidity: Approximately Zero Percent Humidity



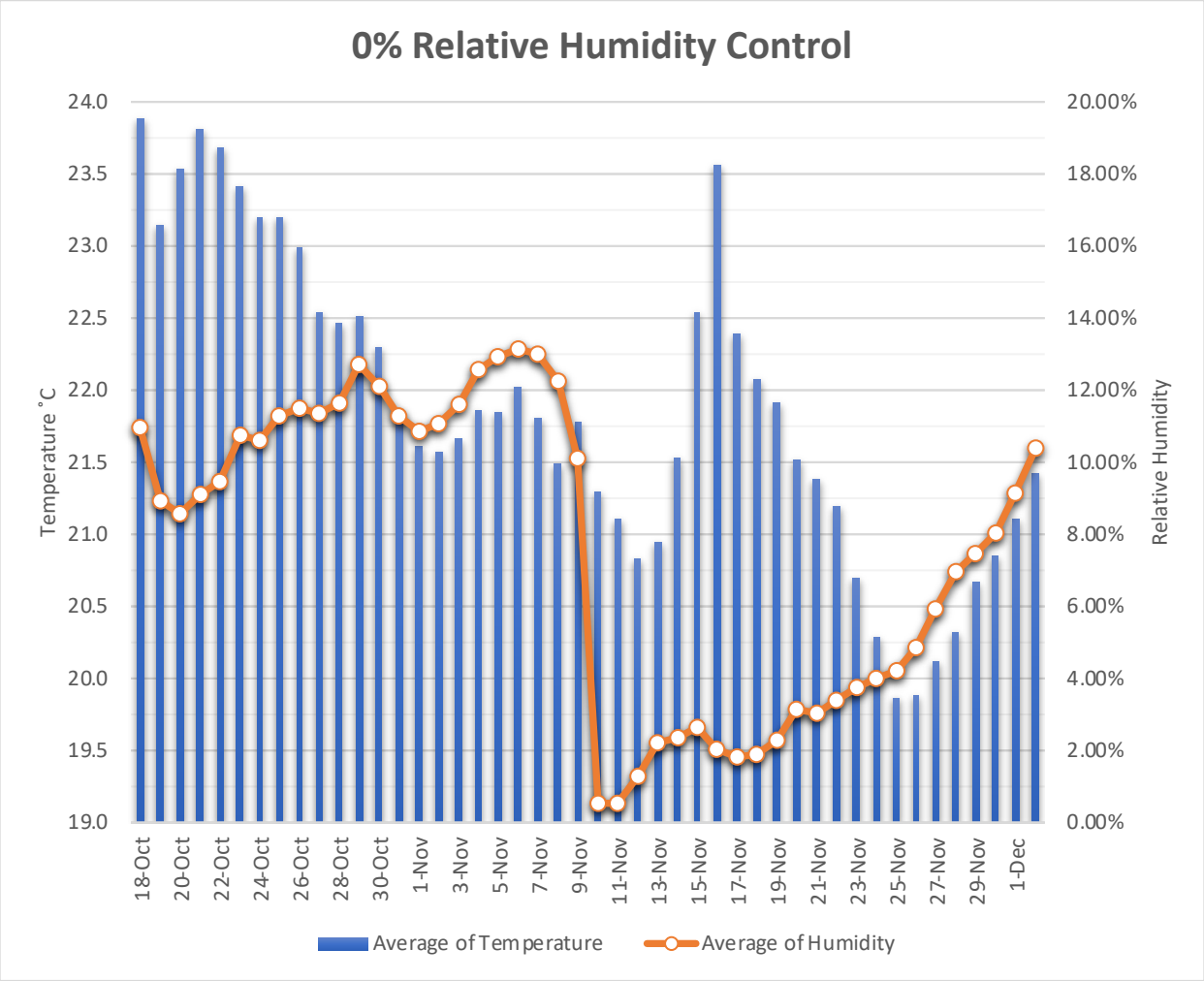
HUMIDITY SUMMARY

Minimum Reading on 10/13/2021 10:20 AM: 1.30%
Maximum Reading on 10/06/2021 03:40 PM: 42.30%
Average Reading: 7.97%

TEMPERATURE SUMMARY

Minimum Reading on 11/12/2021 10:08 AM: 14.20C
Maximum Reading on 10/19/2021 11:43 AM: 30.00C
Average Reading: 18.48C

Humidity: Approximately Zero Percent Humidity Control



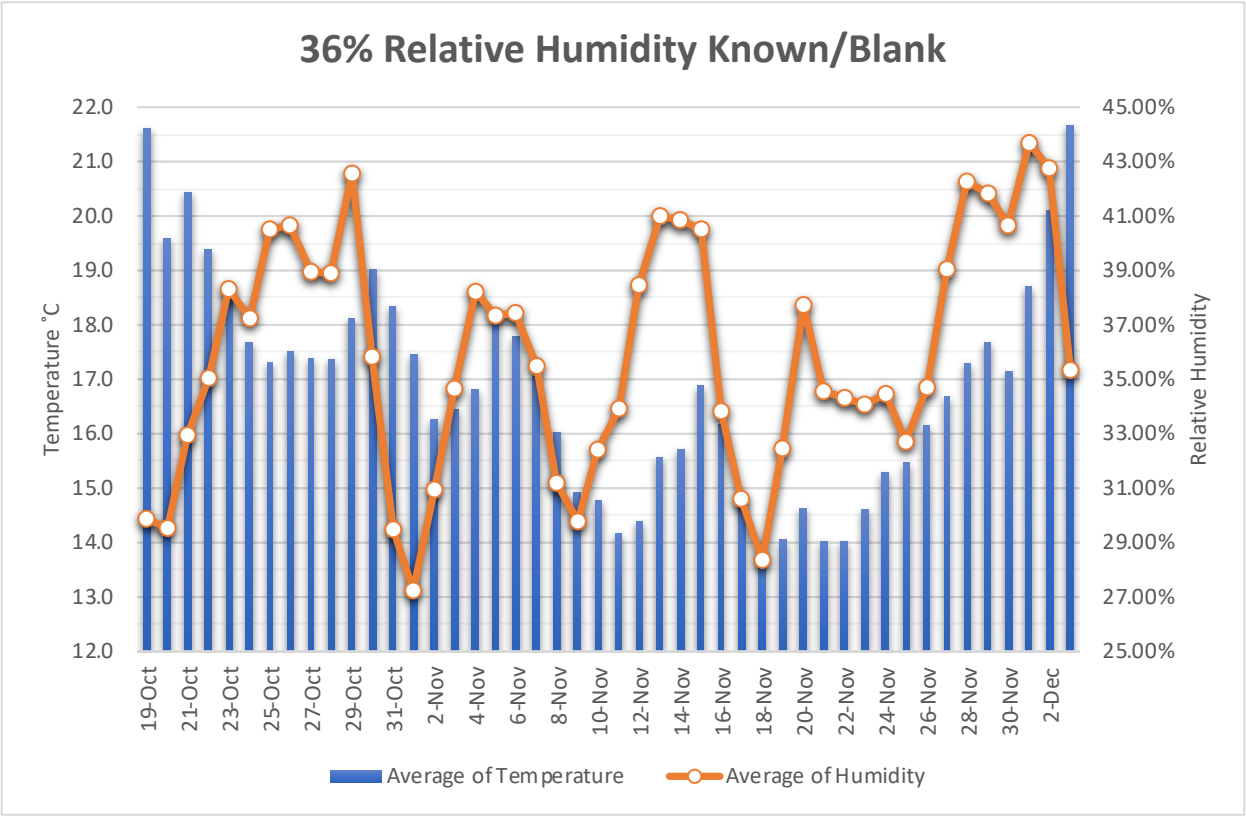
HUMIDITY SUMMARY

Minimum Reading on 11/09/2021 08:45 PM: .20%
Maximum Reading on 11/09/2021 04:41 PM: 46.90%
Average Reading: 7.52%

TEMPERATURE SUMMARY

Minimum Reading on 11/26/2021 01:07 AM: 19.70C
Maximum Reading on 11/09/2021 05:42 PM: 25.90C
Average Reading: 21.84C

Humidity: 36% Humidity



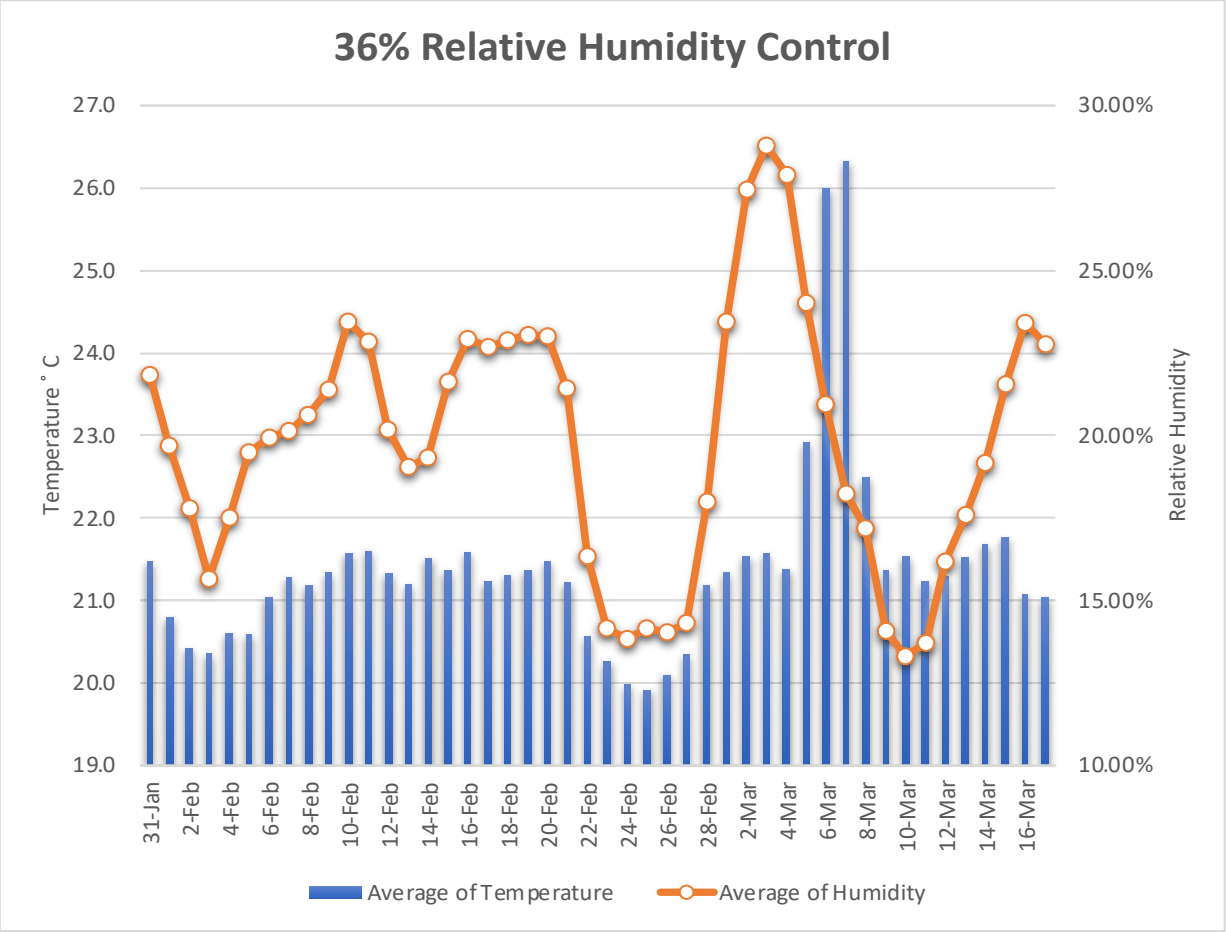
HUMIDITY SUMMARY

Minimum Reading on 10/31/2021 11:45 AM: 15.70%
Maximum Reading on 12/02/2021 07:16 AM: 47.50%
Average Reading: 36.02%

TEMPERATURE SUMMARY

Minimum Reading on 11/22/2021 10:23 AM: 13.10C
Maximum Reading on 10/21/2021 11:49 AM: 35.20C
Average Reading: 16.78C

Humidity: 36% Humidity Control



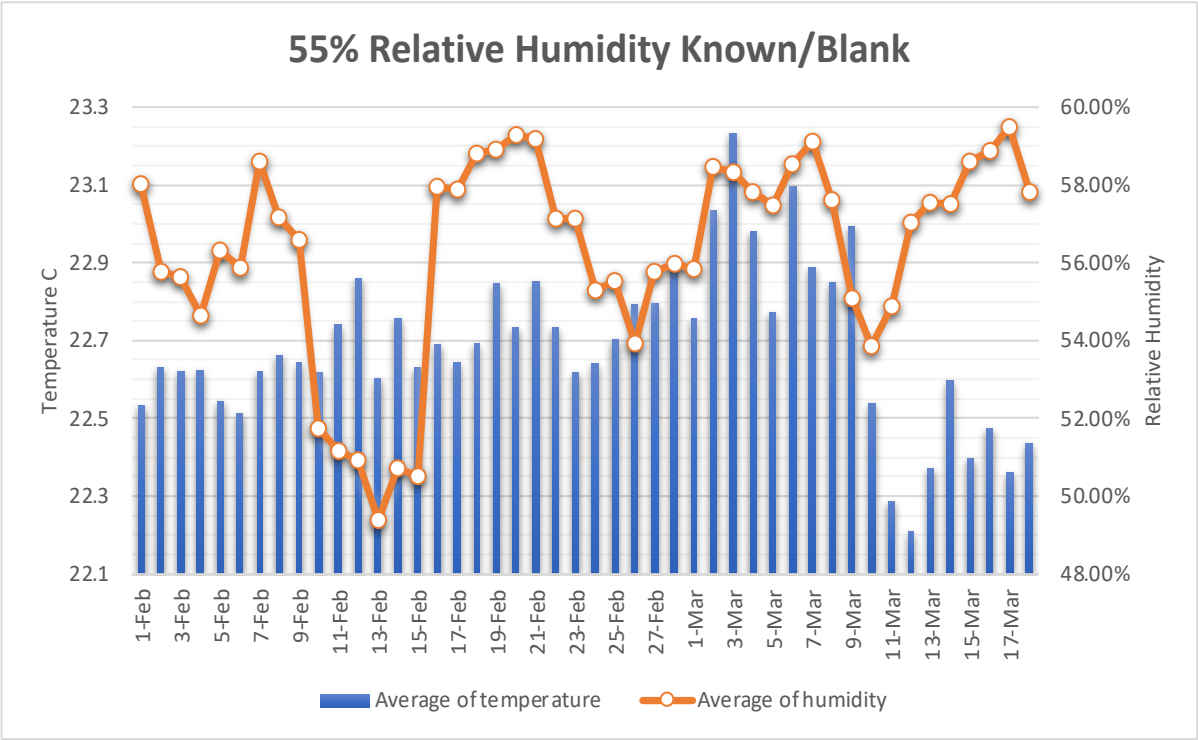
HUMIDITY SUMMARY

Minimum Reading on 03/10/2022 12:01 PM: 13.10%
Maximum Reading on 03/04/2022 02:30 AM: 29.10%
Average Reading: 19.76%

TEMPERATURE SUMMARY

Minimum Reading on 02/24/2022 01:32 PM: 19.70C
Maximum Reading on 03/07/2022 09:48 AM: 27.70C
Average Reading: 21.39C

Humidity: 55% Humidity



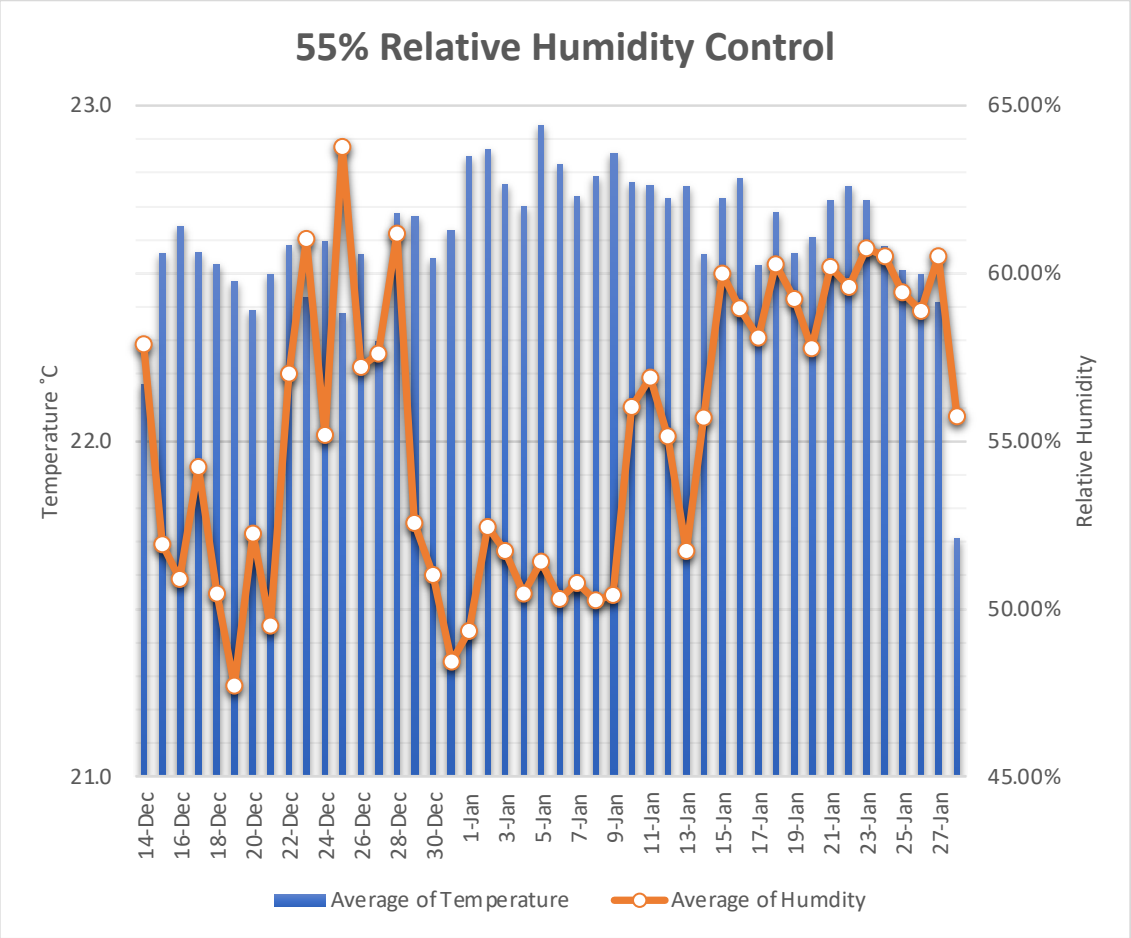
HUMIDITY SUMMARY

Minimum Reading on 02/13/2022 11:18 AM: 44.00%
Maximum Reading on 02/09/2022 05:50 PM: 73.30%
Average Reading: 56.26%

TEMPERATURE SUMMARY

Minimum Reading on 02/01/2022 11:35 AM: 20.40C
Maximum Reading on 03/03/2022 12:12 AM: 23.70C
Average Reading: 22.69C

Humidity: 55% Humidity Control



HUMIDITY SUMMARY

Minimum Reading on 01/28/2022 09:48 AM: 24.40%
Maximum Reading on 12/25/2021 04:20 AM: 80.00%
Average Reading: 55.24%

TEMPERATURE SUMMARY

Minimum Reading on 01/28/2022 09:48 AM: 14.40C
Maximum Reading on 01/02/2022 01:26 AM: 23.60C
Average Reading: 22.62C

Appendix D: qPCR Sample Results

Table 1. Blank, Control, and Known qPCR results.

Sample Name	FAM Cq	FAM Tm	FAM Conc	FAM Exp. Tm?	CO560 Cq	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
50 ng/ul	19.00608	81.6	50	Yes	19.5336565	82.7	50	Yes	21.5341642	N/A	50	No
10 ng/ul	21.2642049	81.5	10	Yes	21.6545429	82.7	10	Yes	21.4743152	N/A	10	No
2 ng/ul	23.9909095	81.4	2	Yes	24.5595666	82.6	2	Yes	21.3071945	N/A	2	No
.4 ng/ul	26.4077533	81.3	0.4	Yes	26.5317288	82.5	0.4	Yes	21.738041	N/A	0.4	No
.08 ng/ul	28.7246898	81.4	0.08	Yes	29.9578929	82.7	0.08	Yes	21.3023713	N/A	0.08	No
.016 ng/ul	31.366363	81.4	0.016	Yes	32.0748114	82.6	0.016	Yes	21.3475963	N/A	0.016	No
.0032 ng/ul	32.2952254	81.5	0.0032	Yes	35.3308191	N/A	0.0032	No Call	21.4311295	N/A	0.003	No
NTC	35.8701562	N/A	0.0006	No	N/A	N/A	N/A	No	21.4862762	N/A	N/A	No
50 ng/ul	18.6562093	81.4	50	Yes	19.2648932	82.6	50	Yes	21.7616072	N/A	50	No
10 ng/ul	20.8876538	81.4	10	Yes	21.4373982	82.5	10	Yes	21.8422047	N/A	10	No
2 ng/ul	24.0664566	81.3	2	Yes	24.4745118	82.4	2	Yes	21.3563368	N/A	2	No
.4 ng/ul	26.155832	81.3	0.4	Yes	26.6129928	82.4	0.4	Yes	21.8792499	N/A	0.4	No
08 ng/ul	28.7596986	81.3	0.08	Yes	29.6414963	82.5	0.08	Yes	21.4196552	N/A	0.08	No

Sample Name	FAM Cq	FAM Tm	FAM Conc	FAM Exp. Tm?	CO560 Cq	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
.016 ng/ul	31.9136777	81.3	0.016	Yes	32.3187659	82.6	0.016	Yes	21.707429	N/A	0.016	No
.0032 ng/ul	32.9725079	81.3	0.0032	Yes	34.294243	N/A	0.0032	No	21.667127	N/A	0.003	No
NTC	36.9216027	N/A	0.0003	No	35.7574259	N/A	0.0017	No	21.5206615	N/A	N/A	No
1	22.3421398	81.4	5.2223	Yes	25.3975772	82.6	1.0634	Yes	22.6990819	N/A	N/A	No
2	21.3788328	81.4	9.9588	Yes	24.4038645	82.6	1.9668	Yes	21.8159852	N/A	N/A	No
3	22.5466429	81.3	4.5535	Yes	24.9681317	82.5	1.3871	Yes	22.6415177	N/A	N/A	No
4	23.8741719	81.3	1.8707	Yes	26.1796205	82.5	0.6554	Yes	21.738941	N/A	N/A	No
5	22.5607676	81.3	4.5106	Yes	24.6838239	82.5	1.6539	Yes	22.0345161	N/A	N/A	No
506	24.0280536	81.4	1.6874	Yes	24.6401727	82.5	1.6992	Yes	22.1398021	N/A	N/A	No
507	24.0816294	81.2	1.6279	Yes	25.4619477	82.5	1.0219	Yes	21.8356208	N/A	N/A	No
508	23.6035411	81.3	2.2427	Yes	24.2493626	82.4	2.1641	Yes	22.0300201	N/A	N/A	No
509	23.3244408	81.2	2.7039	Yes	24.4220229	82.4	1.9448	Yes	21.6888208	N/A	N/A	No
510	23.7720075	81.3	2.0033	Yes	24.828603	82.4	1.5122	Yes	21.3985548	N/A	N/A	No
306	24.7164759	81.4	1.0639	Yes	25.4508022	82.5	1.029	Yes	21.8455485	N/A	N/A	No
307	23.4841863	81.3	2.4294	Yes	24.4136511	82.5	1.9549	Yes	22.0005813	N/A	N/A	No
308	23.5304504	81.3	2.3553	Yes	25.1216106	82.4	1.2614	Yes	21.2811788	N/A	N/A	No
309	24.294804	81.2	1.4112	Yes	25.1705008	82.4	1.2238	Yes	21.719377	N/A	N/A	No
310	25.8151951	81.2	0.5095	Yes	29.0023581	82.4	0.1143	Yes	21.1710505	N/A	N/A	No

Sample Name	FAM Cq	FAM Tm	FAM Conc	FAM Exp. Tm?	CO560 Cq	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
101	23.5504704	81.5	2.3239	Yes	25.2559074	82.7	1.1608	Yes	21.5761353	N/A	N/A	No
102	22.8943538	81.3	3.6071	Yes	24.5794405	82.5	1.7643	Yes	21.8627156	N/A	N/A	No
103	23.347267	81.3	2.6629	Yes	25.3817384	82.4	1.0739	Yes	21.2491759	N/A	N/A	No
104	23.4787913	81.2	2.4382	Yes	24.6959741	82.4	1.6416	Yes	21.7162492	N/A	N/A	No
105	35.138377	81.2	0.001	Yes	36.2532935	N/A	0.0013	No	21.0025289	N/A	N/A	No
401	32.1082108	81.3	0.0075	Yes	34.522743	82.8	0.0038	Yes	21.4277572	N/A	N/A	No
402	33.214172	81.3	0.0036	Yes	33.2266764	82.7	0.0084	Yes	21.8348079	N/A	N/A	No
403	32.2061459	81.2	0.007	Yes	35.0805307	82.6	0.0027	Yes	20.8590765	N/A	N/A	No
404	23.1499301	81.3	3.0393	Yes	25.7789298	82.4	0.8399	Yes	21.5607165	N/A	N/A	No
405	21.9148861	81.3	6.9535	Yes	24.2474465	82.5	2.1667	Yes	21.6441263	N/A	N/A	No
206	32.0393729	N/A	0.0079	No	35.3173617	N/A	0.0023	No	21.5929735	N/A	N/A	No
207	33.6896665	N/A	0.0026	No	34.2006827	N/A	0.0046	No	21.5379096	N/A	N/A	No
208	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.3141332	N/A	N/A	No
209	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.5257541	N/A	N/A	No
210	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.8352724	N/A	N/A	No
216	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.2304215	N/A	N/A	No
217	36.4100471	N/A	0.0004	No	N/A	N/A	N/A	No	21.4852772	N/A	N/A	No
218	30.8305663	N/A	0.0177	No Call	35.5700591	N/A	0.002	No	21.4093472	N/A	N/A	No
219	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.0115941	N/A	N/A	No

Sample Name	FAM Cq	FAM Tm	FAM Conc	FAM Exp. Tm?	CO560 Cq	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
220	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.633298	N/A	N/A	No
B21	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.264953	N/A	N/A	No
B22	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.3511185	N/A	N/A	No
411	N/A	N/A	N/A	No	36.9955813	N/A	0.0008	No	21.707387	N/A	N/A	No
412	32.0390727	N/A	0.0079	No	33.5905822	N/A	0.0067	No	21.2621953	N/A	N/A	No
413	33.2592783	N/A	0.0035	No	N/A	N/A	N/A	No	21.7337132	N/A	N/A	No
414	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.1506713	N/A	N/A	No
415	36.8917141	N/A	0.0003	No	N/A	N/A	N/A	No	21.609839	N/A	N/A	No
B23	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.5769445	N/A	N/A	No
411	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.5863523	N/A	N/A	No
412	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.2559569	N/A	N/A	No
413	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.5652469	N/A	N/A	No
414	34.7125537	N/A	0.0013	No	33.7976806	N/A	0.0059	No	21.2848689	N/A	N/A	No
415	N/A	N/A	N/A	No	37.1316738	N/A	0.0007	No	21.777062	N/A	N/A	No
B24	36.7203827	N/A	0.0003	No	N/A	N/A	N/A	No	21.2850631	N/A	N/A	No

Table 2. Blank Sample's qPCR results.

Sample Name	FAM Cq	FAM Tm	FAM Conc	FAM Exp. Tm?	CO560 Cq	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
50 ng/ul	18.2818	81.6	50	Yes	19.113719	82.8	50	Yes	24.0583681	82	50	Yes
10 ng/ul	21.4198	81.6	10	Yes	22.388452	82.7	10	Yes	22.5811455	81.9	10	Yes
2 ng/ul	23.6345	81.5	2	Yes	24.457492	82.6	2	Yes	22.07546	81.8	2	Yes
.4 ng/ul	25.8532	81.4	0.4	Yes	26.856834	82.5	0.4	Yes	22.5455331	81.7	0.4	Yes
.08 ng/ul	28.6298	81.4	0.08	Yes	29.860439	82.6	0.08	Yes	22.192397	81.7	0.08	Yes
.016 ng/ul	31.6075	81.3	0.016	Yes	32.694752	82.6	0.016	Yes	22.5497871	81.8	0.016	Yes
.0032 ng/ul	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.2422827	81.9	N/A	Yes
NTC	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.6891842	82.1	N/A	Yes
50 ng/ul	19.0325	81.4	50	Yes	19.952321	82.6	50	Yes	24.3074907	81.8	50	Yes
10 ng/ul	21.1395	81.4	10	Yes	21.924542	82.5	10	Yes	23.1313927	81.7	10	Yes
2 ng/ul	23.7697	81.4	2	Yes	24.307407	82.5	2	Yes	22.8106038	81.7	2	Yes
.4 ng/ul	26.3935	81.3	0.4	Yes	26.697794	82.4	0.4	Yes	22.0396855	81.7	0.4	Yes
.08 ng/ul	28.9187	81.3	0.08	Yes	29.642685	82.5	0.08	Yes	22.7796623	81.6	0.08	Yes
.016 ng/ul	N/A	N/A	0.016	No	N/A	N/A	0.016	No	22.1373621	81.7	0.016	Yes
.0032 ng/ul	32.729	81.2	0.006749	Yes	34.247914	N/A	0.0046	No Call	22.1191807	81.8	N/A	Yes
NTC	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.2740543	81.9	N/A	Yes
11	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.3654402	81.7	N/A	Yes

Sample Name	FAM Cq	FAM Tm	FAM Conc	FAM Exp. Tm?	CO560 Cq	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
12	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.7417653	81.7	N/A	Yes
13	32.7921	N/A	0.006485	No	N/A	N/A	N/A	No	22.9679839	81.7	N/A	Yes
14	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.3457652	81.6	N/A	Yes
15	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.7963923	81.6	N/A	Yes
11	36.2023	N/A	0.00075	No	N/A	N/A	N/A	No	22.5955455	81.7	N/A	Yes
12	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.2630928	81.7	N/A	Yes
13	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.7994688	81.6	N/A	Yes
14	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.6184537	81.5	N/A	Yes
15	N/A	N/A	N/A	No	36.355842	N/A	0.0012	No	22.6465192	81.6	N/A	Yes
516	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.352271	81.8	N/A	Yes
517	30.766	N/A	0.023362	No	N/A	N/A	N/A	No	22.3935129	81.7	N/A	Yes
518	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.4689826	81.7	N/A	Yes
519	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.6525603	81.6	N/A	Yes
520	29.767	N/A	0.043949	No	N/A	N/A	N/A	No	22.0685316	81.5	N/A	Yes
516	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.6853231	81.8	N/A	Yes
517	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.938654	81.8	N/A	Yes
518	34.7638	N/A	0.001863	No	N/A	N/A	N/A	No	22.0749151	81.7	N/A	Yes
519	32.7976	N/A	0.006462	No	N/A	N/A	N/A	No	22.7985063	81.6	N/A	Yes
520	33.5113	N/A	0.004114	No	N/A	N/A	N/A	No	22.3131231	81.7	N/A	Yes
316	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.4074184	81.9	N/A	Yes
317	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.916063	81.8	N/A	Yes
318	36.9133	N/A	0.000478	No	N/A	N/A	N/A	No	22.0141969	81.7	N/A	Yes
319	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.8631599	81.6	N/A	Yes
320	35.1159	N/A	0.001491	No	N/A	N/A	N/A	No	22.6650973	81.7	N/A	Yes

Sample Name	FAM Cq	FAM Tm	FAM Conc	FAM Exp. Tm?	CO560 Cq	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
316	35.758	N/A	0.000993	No	N/A	N/A	N/A	No	22.5118279	81.8	N/A	Yes
317	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.7477495	81.7	N/A	Yes
318	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.2829419	81.6	N/A	Yes
319	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.0820107	81.6	N/A	Yes
320	32.6287	N/A	0.007191	No	N/A	N/A	N/A	No	22.5542297	81.6	N/A	Yes
111	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.5746696	81.8	N/A	Yes
112	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.1558218	81.6	N/A	Yes
113	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.3619121	81.7	N/A	Yes
114	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.3387916	81.7	N/A	Yes
115	34.5388	N/A	0.002148	No	N/A	N/A	N/A	No	22.4221663	81.7	N/A	Yes
111	N/A	N/A	N/A	No	37.821615	N/A	0.0005	No	22.6900496	81.8	N/A	Yes
112	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.1698882	81.7	N/A	Yes
113	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.4184347	81.7	N/A	Yes
114	34.8492	N/A	0.001765	No	N/A	N/A	N/A	No	22.1898207	81.6	N/A	Yes
115	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.4653354	81.6	N/A	Yes
211	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.7725037	81.8	N/A	Yes
212	32.3418	N/A	0.008622	No	N/A	N/A	N/A	No	22.1957272	81.8	N/A	Yes
213	N/A	N/A	N/A	No	37.56884	N/A	0.0006	No	22.3694191	81.6	N/A	Yes
214	37.8104	N/A	0.000271	No	N/A	N/A	N/A	No	22.1097549	81.7	N/A	Yes
215	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.1911926	81.7	N/A	Yes
211	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.6535061	81.9	N/A	Yes
212	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.4597617	81.8	N/A	Yes
213	37.6325	N/A	0.000303	No	N/A	N/A	N/A	No	22.4779144	81.7	N/A	Yes
214	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.8992021	81.7	N/A	Yes

Sample Name	FAM Cq	FAM Tm	FAM Conc	FAM Exp. Tm?	CO560 Cq	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
215	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.6728221	81.6	N/A	Yes

Table 3. Known Sample's qPCR results.

Sample Name	FAM Cq	FAM Tm	FAM Conc	FAM Exp. Tm?	CO560 Cq	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
50 ng/ul	18.1542456	N/A	50	No	18.8915262	N/A	50	No	21.4100615	N/A	50	No
10 ng/ul	21.0051464	N/A	10	No	21.6184372	N/A	10	No	21.9806800	N/A	10	No
2 ng/ul	24.1301842	N/A	2	No	24.2325765	N/A	2	No	21.3560578	N/A	2	No
.4 ng/ul	26.6106838	N/A	0.4	No	27.0111326	N/A	0.4	No	21.6033765	N/A	0.4	No
.08 ng/ul	29.0051227	N/A	0.08	No	29.7601688	N/A	0.08	No	21.3738462	N/A	0.08	No
.016 ng/ul	31.8064853	N/A	0.016	No	32.8278429	N/A	0.016	No	22.1235940	N/A	0.016	No
.0032 ng/ul	32.8779314	N/A	0.0074	No	N/A	N/A	N/A	No	21.7586678	N/A	N/A	No
NTC	33.8155071	N/A	0.0042	No	N/A	N/A	N/A	No	21.7993955	N/A	N/A	No
50 ng/ul	18.2497103	N/A	50	No	19.1765392	N/A	50	No	21.9317994	N/A	50	No
10 ng/ul	21.0538090	N/A	10	No	21.2297718	N/A	10	No	22.0616050	N/A	10	No
2 ng/ul	23.5743649	N/A	2	No	23.7531264	N/A	2	No	21.5732306	N/A	2	No
.4 ng/ul	26.2989066	N/A	0.4	No	26.5999814	N/A	0.4	No	21.9929549	N/A	0.4	No
.08 ng/ul	28.2340918	N/A	0.08	No	29.7863426	N/A	0.08	No	21.8282057	N/A	0.08	No
.016 ng/ul	31.5557364	N/A	0.016	No	32.3780082	N/A	0.016	No	21.5379651	N/A	0.016	No

Sample Name	FAM Cq	FAM Tm	FAM Conc	FAM Exp. Tm?	CO560 Cq	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
.0032 ng/ul	32.2645396	N/A	0.0107	No	34.1657818	N/A	0.005799	No	22.2453125	N/A	N/A	No
NTC	32.5148597	N/A	0.0092	No	37.8950983	N/A	0.000646	No	22.6523871	N/A	N/A	No
201	32.3672889	N/A	0.01	No	34.7436080	N/A	0.004127	No	22.4901672	N/A	N/A	No
202	22.2961158	N/A	4.5448	No	24.7065464	N/A	1.51818	No	22.3721089	N/A	N/A	No
203	23.7171656	N/A	1.9177	No	26.3093622	N/A	0.591034	No	23.3267025	N/A	N/A	No
204	24.5977004	N/A	1.1235	No	26.2175241	N/A	0.623862	No	21.8459824	N/A	N/A	No
205	23.0587130	N/A	2.8603	No	24.0684462	N/A	2.210212	No	22.1915497	N/A	N/A	No
201	32.1659220	N/A	0.0113	No	35.0728421	N/A	0.0034	No	24.0632108	N/A	N/A	No
202	22.6916316	N/A	3.5745	No	25.2847412	N/A	1.080253	No	22.1080545	N/A	N/A	No
203	23.3190639	N/A	2.4421	No	25.4828598	N/A	0.961352	No	22.3672326	N/A	N/A	No
204	26.1355452	N/A	0.4416	No	30.3985354	N/A	0.053253	No	26.0088175	N/A	N/A	No
205	23.4432719	N/A	2.2647	No	24.6399476	N/A	1.578873	No	21.9617322	N/A	N/A	No

Table 4. Control Sample's 1st Run qPCR results.

Sample Name	FAM Cq	FAM Tm	FAM Tm2	FAM Conc	FAM Exp. Tm?	FAM Exp. Tm2?	CO560 Cq	CO560 Tm	CO560 Tm2	CO560 Conc	CO560 Exp. Tm?	CO560 Exp. Tm2?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
50 ng/ul	18.7	N/A	81.5	50	No	Yes	19.7	N/A	82.7	50	No	Yes	22.7	N/A	50	No
10 ng/ul	21.2	N/A	81.5	10	No	Yes	22.0	N/A	82.7	10	No	Yes	22.2	N/A	10	No
2 ng/ul	23.7	N/A	81.4	2	No	Yes	24.2	N/A	82.6	2	No	Yes	22.0	N/A	2	No
.4 ng/ul	26.3	N/A	81.4	0.4	No	Yes	27.0	N/A	82.5	0.4	No	Yes	22.0	N/A	0.4	No
.08 ng/ul	28.6	N/A	81.3	0.080	No	Yes	29.8	N/A	82.5	0.080	No	Yes	22.2	N/A	0.08	No
.016 ng/ul	31.6	N/A	N/A	0.016	No	No	32.8	N/A	82.8	0.016	No Call	Yes	22.2	N/A	0.016	No
.0032 ng/ul	32.4	N/A	N/A	0.010	No	No	33.9	N/A	82.8	0.006	No Call	Yes	22.4	N/A	N/A	No
NTC	32.5	N/A	N/A	0.009	No Call	No	37.0	N/A	N/A	0.001	No	No	22.2	N/A	N/A	No
50 ng/ul	18.3	N/A	81.4	50.000	No	Yes	19.4	N/A	82.6	50.000	No	Yes	22.6	N/A	50	No
10 ng/ul	20.8	N/A	81.4	10.000	No	Yes	21.3	N/A	82.6	10.000	No	Yes	22.3	N/A	10	No
2 ng/ul	23.4	N/A	81.3	2.000	No	Yes	23.8	N/A	82.5	2.000	No	Yes	22.2	N/A	2	No
.4 ng/ul	26.0	N/A	81.3	0.400	No	Yes	26.4	N/A	82.4	0.400	No	Yes	21.9	N/A	0.4	No
.08 ng/ul	28.9	N/A	81.3	0.080	No	Yes	29.6	N/A	82.5	0.080	No	Yes	22.2	N/A	0.08	No

Sample Name	FAM Cq	FAM Tm	FAM Tm2	FAM Conc	FAM Exp. Tm?	FAM Exp. Tm2?	CO560 Cq	CO560 Tm	CO560 Tm2	CO560 Conc	CO560 Exp. Tm?	CO560 Exp. Tm2?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
.016 ng/ul	32.0	N/A	80.9	0.016	No	Yes	32.2	N/A	82.6	0.016	No Call	Yes	22.3	N/A	0.016	No
.0032 ng/ul	31.6	78.8	N/A	0.016	Yes	No	37.7	N/A	N/A	0.001	No	No	22.1	N/A	N/A	No
NTC	30.7	79.2	N/A	0.028	Yes	No	35.5	N/A	N/A	0.002	No Call	No	22.4	N/A	N/A	No
12	30.6	78.5	N/A	0.030	Yes	No	34.7	N/A	N/A	0.003	No Call	No	22.2	N/A	N/A	No
67	30.4	N/A	N/A	0.033	No Call	No	35.0	N/A	N/A	0.003	No	No	21.4	N/A	N/A	No
52	32.0	N/A	N/A	0.012	No Call	No	34.5	N/A	N/A	0.004	No Call	No	21.9	N/A	N/A	No
30	31.1	78.6	N/A	0.022	Yes	No	34.4	N/A	N/A	0.004	No Call	No	22.1	N/A	N/A	No
37	31.1	N/A	N/A	0.021	No Call	No	35.3	N/A	N/A	0.002	No	No	23.6	N/A	N/A	No
65	31.2	N/A	N/A	0.020	No Call	No	35.3	N/A	N/A	0.002	No	No	22.1	N/A	N/A	No
80	32.4	N/A	N/A	0.010	No	No	34.5	N/A	N/A	0.004	No	No	21.7	N/A	N/A	No
45	32.1	N/A	N/A	0.011	No	No	36.2	N/A	N/A	0.001	No	No	22.3	N/A	N/A	No
17	31.9	N/A	N/A	0.013	No Call	No	35.0	N/A	N/A	0.003	No Call	No	22.1	N/A	N/A	No
15	32.4	N/A	N/A	0.010	No	No	35.3	N/A	N/A	0.002	No Call	No	22.2	N/A	N/A	No
501	31.1	N/A	N/A	0.021	No Call	No	36.0	N/A	N/A	0.002	No	No	22.0	N/A	N/A	No

Sample Name	FAM Cq	FAM Tm	FAM Tm2	FAM Conc	FAM Exp. Tm?	FAM Exp. Tm2?	CO560 Cq	CO560 Tm	CO560 Tm2	CO560 Conc	CO560 Exp. Tm?	CO560 Exp. Tm2?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
502	30.3	78.6	N/A	0.035	Yes	No	N/A	N/A	N/A	N/A	No	No	22.2	N/A	N/A	No
503	31.0	78.3	N/A	0.023	Yes	No	35.3	N/A	N/A	0.002	No	No	22.2	N/A	N/A	No
504	30.6	78.3	N/A	0.029	Yes	No	35.3	N/A	N/A	0.002	No	No	22.1	N/A	N/A	No
505	31.2	78.6	N/A	0.020	Yes	No	35.2	N/A	N/A	0.003	No	No	22.0	N/A	N/A	No
511	32.2	78.8	N/A	0.011	Yes	No	N/A	N/A	N/A	N/A	No	No	22.2	N/A	N/A	No
512	31.2	N/A	N/A	0.020	No Call	No	35.8	N/A	N/A	0.002	No Call	No	22.1	N/A	N/A	No
513	32.8	N/A	N/A	0.007	No Call	No	34.7	N/A	N/A	0.003	No Call	No	22.0	N/A	N/A	No
514	33.0	78.8	N/A	0.007	Yes	No	34.3	78	N/A	0.004	Yes	No	22.1	N/A	N/A	No
515	33.2	78.9	N/A	0.006	Yes	No	36.3	N/A	N/A	0.001	No Call	No	21.9	N/A	N/A	No
301	30.8	79	N/A	0.026	Yes	No	34.4	N/A	N/A	0.004	No Call	No	22.3	N/A	N/A	No
302	32.0	N/A	N/A	0.012	No Call	No	34.2	77.6	N/A	0.005	Yes	No	22.2	N/A	N/A	No
303	32.6	78.7	N/A	0.008	Yes	No	35.1	N/A	N/A	0.003	No	No	22.2	N/A	N/A	No
304	32.6	79.2	N/A	0.009	Yes	No	36.2	N/A	N/A	0.001	No	No	21.4	N/A	N/A	No
305	30.0	N/A	N/A	0.041	No	No	35.6	N/A	N/A	0.002	No Call	No	22.0	N/A	N/A	No
311	32.5	N/A	N/A	0.009	No Call	No	36.2	N/A	N/A	0.001	No Call	No	22.2	N/A	N/A	No
312	32.3	78.9	N/A	0.010	Yes	No	35.6	N/A	N/A	0.002	No Call	No	22.1	N/A	N/A	No
313	32.7	N/A	N/A	0.008	No	No	36.0	N/A	N/A	0.002	No	No	22.1	N/A	N/A	No
314	31.9	78.6	N/A	0.013	Yes	No	36.8	N/A	N/A	0.001	No	No	22.1	N/A	N/A	No

Sample Name	FAM Cq	FAM Tm	FAM Tm2	FAM Conc	FAM Exp. Tm?	FAM Exp. Tm2?	CO560 Cq	CO560 Tm	CO560 Tm2	CO560 Conc	CO560 Exp. Tm?	CO560 Exp. Tm2?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
315	31.9	N/A	N/A	0.013	No Call	No	36.7	N/A	N/A	0.001	No	No	22.1	N/A	N/A	No
106	32.9	79.2	N/A	0.007	Yes	No	36.2	N/A	N/A	0.001	No	No	22.0	N/A	N/A	No
107	32.1	79.1	N/A	0.011	Yes	No	36.5	N/A	N/A	0.001	No	No	21.6	N/A	N/A	No
108	29.9	79	N/A	0.046	Yes	No	35.1	N/A	N/A	0.003	No	No	21.4	N/A	N/A	No
109	32.3	78.5	N/A	0.010	Yes	No	33.5	N/A	N/A	0.007	No Call	No	22.2	N/A	N/A	No
110	31.7	78.7	N/A	0.015	Yes	No	35.6	N/A	N/A	0.002	No Call	No	21.8	N/A	N/A	No
116	32.3	N/A	N/A	0.010	No	No	34.8	77.7	N/A	0.003	Yes	No	22.3	N/A	N/A	No
117	31.5	N/A	N/A	0.017	No Call	No	34.6	N/A	N/A	0.004	No Call	No	21.9	N/A	N/A	No
118	31.3	N/A	N/A	0.019	No Call	No	N/A	N/A	N/A	N/A	No	No	22.0	N/A	N/A	No
119	31.6	N/A	N/A	0.016	No	No	35.0	N/A	N/A	0.003	No	No	22.0	N/A	N/A	No
120	30.8	78.7	N/A	0.025	Yes	No	36.3	N/A	N/A	0.001	No Call	No	21.8	N/A	N/A	No
406	31.5	79.2	N/A	0.016	Yes	No	34.8	N/A	N/A	0.003	No Call	No	22.0	N/A	N/A	No
407	32.1	79.1	N/A	0.011	Yes	No	36.1	N/A	N/A	0.001	No Call	No	22.1	N/A	N/A	No
408	33.0	N/A	N/A	0.007	No Call	No	N/A	N/A	N/A	N/A	No	No	21.9	N/A	N/A	No
409	32.1	N/A	N/A	0.011	No Call	No	35.2	N/A	N/A	0.003	No Call	No	22.6	N/A	N/A	No

Sample Name	FAM Cq	FAM Tm	FAM Tm2	FAM Conc	FAM Exp. Tm?	FAM Exp. Tm2?	CO560 Cq	CO560 Tm	CO560 Tm2	CO560 Conc	CO560 Exp. Tm?	CO560 Exp. Tm2?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
410	32.3	N/A	N/A	0.010	No Call	No	35.1	N/A	N/A	0.003	No Call	No	22.2	N/A	N/A	No
416	31.1	78.9	N/A	0.022	Yes	No	35.4	N/A	N/A	0.002	No Call	No	22.2	N/A	N/A	No
417	31.7	N/A	N/A	0.015	No Call	No	36.7	N/A	N/A	0.001	No Call	No	22.5	N/A	N/A	No
418	29.4	N/A	N/A	0.061	No Call	No	35.8	N/A	N/A	0.002	No Call	No	22.4	N/A	N/A	No
419	31.8	79.1	N/A	0.014	Yes	No	36.0	N/A	N/A	0.002	No Call	No	22.0	N/A	N/A	No
420	31.4	79.1	N/A	0.017	Yes	No	35.8	N/A	N/A	0.002	No	No	22.0	N/A	N/A	No
B1	32.9	79.1	N/A	0.007	Yes	No	36.8	N/A	N/A	0.001	No	No	22.1	N/A	N/A	No
B2	31.8	78.9	N/A	0.014	Yes	No	36.1	N/A	N/A	0.001	No Call	No	22.0	N/A	N/A	No
B3	32.5	78.9	N/A	0.009	Yes	No	36.2	N/A	N/A	0.001	No Call	No	22.2	N/A	N/A	No
B4	32.9	N/A	N/A	0.007	No Call	No	36.2	N/A	N/A	0.001	No Call	No	22.2	N/A	N/A	No
B5	32.5	N/A	N/A	0.009	No Call	No	35.5	N/A	N/A	0.002	No Call	No	22.4	N/A	N/A	No
B6	32.1	79	N/A	0.012	Yes	No	35.8	N/A	N/A	0.002	No Call	No	22.1	N/A	N/A	No
B7	31.2	N/A	N/A	0.020	No Call	No	36.1	N/A	N/A	0.001	No	No	21.6	N/A	N/A	No
B8	31.4	N/A	N/A	0.018	No Call	No	34.7	N/A	N/A	0.003	No Call	No	21.7	N/A	N/A	No

Sample Name	FAM Cq	FAM Tm	FAM Tm2	FAM Conc	FAM Exp. Tm?	FAM Exp. Tm2?	CO560 Cq	CO560 Tm	CO560 Tm2	CO560 Conc	CO560 Exp. Tm?	CO560 Exp. Tm2?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
B9	31.9	N/A	N/A	0.013	No Call	No	35.5	N/A	N/A	0.002	No	No	21.8	N/A	N/A	No
B10	30.4	78.6	N/A	0.033	Yes	No	N/A	N/A	N/A	N/A	No	No	21.9	N/A	N/A	No
B11	30.9	78	N/A	0.024	Yes	No	35.3	77.8	N/A	0.002	Yes	No	22.3	N/A	N/A	No
B12	32.4	79.1	N/A	0.010	Yes	No	34.7	N/A	N/A	0.004	No Call	No	22.2	N/A	N/A	No
B13	30.2	N/A	80.4	0.037	No	Yes	34.6	N/A	N/A	0.004	No	No Call	22.2	N/A	N/A	No
B14	32.0	N/A	N/A	0.012	No	No	35.3	N/A	N/A	0.002	No Call	No	22.0	N/A	N/A	No
B15	31.5	N/A	N/A	0.016	No Call	No	36.7	N/A	N/A	0.001	No	No	22.2	N/A	N/A	No
B16	31.0	78.7	N/A	0.023	Yes	No	34.6	N/A	N/A	0.004	No Call	No	21.8	N/A	N/A	No
B17	31.1	78.8	N/A	0.022	Yes	No	35.5	N/A	N/A	0.002	No	No	22.1	N/A	N/A	No
B18	31.1	N/A	N/A	0.021	No Call	No	36.6	N/A	N/A	0.001	No	No	22.1	N/A	N/A	No
B19	31.9	N/A	N/A	0.013	No Call	No	N/A	N/A	N/A	N/A	No	No	22.4	N/A	N/A	No
B20	31.7	78.7	N/A	0.015	Yes	No	36.3	N/A	N/A	0.001	No	No	22.0	N/A	N/A	No

Table 5. Control Sample's 2nd Run qPCR results.

Sample Name	FAM Cq	FAM Tm	FAM Conc	FAM Exp. Tm?	CO560 Cq	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
50 ng/ul	19.33657	81.6	50	Yes	19.50868	82.7	50	Yes	22.16691	N/A	50	No
10 ng/ul	22.03742	81.6	10	Yes	22.17852	82.7	10	Yes	21.66352	N/A	10	No
2 ng/ul	24.09619	81.5	2	Yes	24.40893	82.6	2	Yes	21.6765	N/A	2	No
.4 ng/ul	28.90449	81.4	0.4	Yes	29.32016	82.7	0.4	Yes	21.49541	N/A	0.4	No
.08 ng/ul	31.20345	81.4	0.08	Yes	32.05321	82.7	0.08	Yes	21.62339	N/A	0.08	No
.016 ng/ul	35.96197	N/A	0.016	No	34.85549	N/A	0.016	No Call	21.9145	N/A	0.016	No
.0032 ng/ul	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.51072	N/A	N/A	No
NTC	36.87703	N/A	0.005451	No	N/A	N/A	N/A	No	21.60708	N/A	N/A	No
50 ng/ul	19.4974	81.6	50	Yes	19.3234	82.6	50	Yes	22.20186	N/A	50	No
10 ng/ul	21.7378	81.4	10	Yes	21.96197	82.5	10	Yes	21.61546	N/A	10	No
2 ng/ul	23.95275	81.4	2	Yes	23.95548	82.6	2	Yes	21.66935	N/A	2	No
.4 ng/ul	28.6126	81.3	0.4	Yes	28.57566	82.6	0.4	Yes	21.95827	N/A	0.4	No
.08 ng/ul	31.40086	81.4	0.08	Yes	31.63532	82.9	0.08	Yes	21.96632	N/A	0.08	No
.016 ng/ul	34.37612	81.3	0.016	Yes	32.3947	82.8	0.016	Yes	21.46776	N/A	0.016	No
.0032ng/ul	N/A	N/A	N/A	No	36.54423	N/A	0.004533	No	22.0738	N/A	N/A	No
NTC	33.85872	N/A	0.024963	No	34.74716	N/A	0.011873	No	21.58146	N/A	N/A	No
45	37.02795	N/A	0.005052	No	33.04422	N/A	0.02957	No	22.17662	N/A	N/A	No
12	N/A	N/A	N/A	No	36.92041	N/A	0.003706	No	21.35293	N/A	N/A	No
52	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.84182	N/A	N/A	No
37	36.52518	N/A	0.006509	No	N/A	N/A	N/A	No	21.30035	N/A	N/A	No
311	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.69835	N/A	N/A	No
315	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.41867	N/A	N/A	No
303	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.614	N/A	N/A	No
304	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.40999	N/A	N/A	No

Sample Name	FAM Cq	FAM Tm	FAM Conc	FAM Exp. Tm?	CO560 Cq	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Cq	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
501	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.12657	N/A	N/A	No
511	36.03396	N/A	0.008338	No	N/A	N/A	N/A	No	21.48931	N/A	N/A	No
513	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.13299	N/A	N/A	No
504	N/A	N/A	N/A	No	36.51807	N/A	0.004597	No	22.11583	N/A	N/A	No
106	N/A	N/A	N/A	No	37.07088	N/A	0.003419	No	22.00545	N/A	N/A	No
108	N/A	N/A	N/A	No	37.65057	N/A	0.002506	No	21.17362	N/A	N/A	No
120	N/A	N/A	N/A	No	37.92546	N/A	0.002163	No	21.24414	N/A	N/A	No
119	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.03329	N/A	N/A	No
416	37.25396	N/A	0.004508	No	N/A	N/A	N/A	No	21.59807	N/A	N/A	No
418	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.45715	N/A	N/A	No
409	37.20754	N/A	0.004614	No	N/A	N/A	N/A	No	21.63544	N/A	N/A	No
410	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.12253	N/A	N/A	No
B5	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.36564	N/A	N/A	No
B10	N/A	N/A	N/A	No	N/A	N/A	N/A	No	22.10537	N/A	N/A	No
B15	37.00438	N/A	0.005112	No	34.56236	N/A	0.013109	No	21.07928	N/A	N/A	No