QUANTITATION OF DNA FROM WAX-EMBEDDED BIOPSY TISSUES USING TWO DIFFERENT METHODS

By

LAURIE ARIOLA

Bachelor of Science in Biochemistry & Molecular Biology

Oklahoma State University

Stillwater, OK

2008

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July 2012

QUANTITATION OF DNA FROM WAX-EMBEDDED BIOPSY TISSUES USING TWO DIFFERENT METHODS

Thesis Approved:

Robert Allen, Ph.D.

Thesis Adviser

Karlis Sloka, D.O

Committee Member

Ronald Thrasher, Ph.D.

Committee Member

David Wallace Ph.D.

Committee Member

Dr. Sheryl A. Tucker

Dean of the Graduate College

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

Allele	The specific form a gene takes at a given position on the chromosome, often one of two forms.
AMEL	Amelogenin, referring to the X and Y amelogenin loci identified the Q-TAT method.
Amplification	The process of increasing starting DNA to a workable quantity, often using PCR.
Bp	Base pair.
CAP	College of American Pathologists
C_T	The threshold cycle, or a cycle in the exponential phase of Real Time PCR at which the software begins quantitating the amount of DNA present in a sample.
DEXPAT	A reagent designed by Takara Bio Inc (Shiga, Japan) to streamline DNA extraction from paraffin embedded samples.
DI water	Stands for deionized water, meaning that ions found in tap water have been removed. Commonly used in chemical procedures.

DNA	Stands for deoxyribonucleic acid, the hereditary material in living organisms. The individuality of DNA among humans allows for identification with STR profiling.
dNTP	Stands for deoxyribonucleotide, a single unit of DNA. A dNTP mixture is added to PCR reactions to facilitate the extension of template DNA.
Efficiency	A measure of the rate and productivity of the PCR reaction.
End-point PCR	A PCR method that quantitates DNA after completion of the amplification process.
FAM	A fluorescent dye used to visualize DNA segments.
FFPE	Stands for "formalin-fixed and paraffin-embedded".
H&E	Stands for Hematoxylin and eosin. H&E is a common histological stain used on biopsy sections to make pathologic diagnoses.
μl	Microliter, or $1x10^{-6}$ liters.
μm	Micrometer, micron, or $1x10^{-6}$ meters.
mtDNA	Mitochondrial DNA, or DNA found in the mitochondria of the cell.

nDNA	Nuclear DNA.
NED	A fluorescent dye used to visualize DNA segments.
OSU	Oklahoma State University
PCR	Stands for Polymerase Chain Reaction. By adding specific chemicals to a PCR run, DNA can be 'amplified' from a small amount that is insufficient for analysis to a larger quantity that can be successfully examined. This technique is a widely used method for increasing the amount of DNA a sample contains.
pRL	A plasmid found in *Renila rentiformis* that contains a gene encoding luciferase. Used in Q-TAT to determine if inhibition is present.
Q-TAT	Stands for Quantitative Template Assay Technology, and end-point PCR method developed at OSU Center for Health Sciences.
qPCR	See *Real Time PCR*.
R^2	The value that describes how well points on a standard curve plot adhere to a line of best fit. A value of 1.00 indicates a perfect fit.
Reagent	A chemical that is added to a process to bring about a specific result.

Real Time PCR	A PCR method that quantitates DNA during the amplification procedure, or in "real time".
RFU	Relative Fluorescence Units.
RNA	Stands for Ribonucleic Acid and, like DNA, encodes genetic information. RNA is a single stranded molecule and carries out many different functions within the cell.
STR	Stands for Short Tandem Repeat. STRs are extremely variable regions of DNA used to compile human genetic profiles.
Supernatant	Liquid on the surface of a mixture. Typically suspended over a solid (the precipitate), the supernatant can also consist of the upper of two liquid layers after centrifugation.
Template	Here, the original strand of DNA to be amplified in PCR.
Quantitation Method	A scientific technique used to determine how much of a substance exists; here, DNA.

Chapter I

Introduction

Millions of biopsies are performed each year, many leading to life-altering diagnoses. As a result of multi-phase processing and handling by different individuals, biopsy samples that form the basis for these diagnoses are subject to contamination. While error is expected in human tasks, every precaution should be taken to minimize and safeguard against it. Identifying contamination or other errors when they do occur is of the utmost importance. To that end, this study will seek to accomplish two goals: The first is to reliably and accurately extract DNA from biopsy tissues for identification purposes, and the second to compare two quantitation methods to examine which is more suitable for quantitating these DNA extracts.

Discovering a contamination event is challenging on many levels, and as such, exact rates of occurrence are hard to pin down. In a 1996 study conducted by the College of American Pathologists (CAP), contamination was estimated to occur in up to 22% of cases.[1] Lab procedures have improved in the years since the CAP study was published, but foreign tissue in biopsy sections is nevertheless still a concern.[2] Some

forms of cross-contamination do not result in an incorrect diagnosis, and therefore will not indicate an error has occurred. However, the contamination of tissue from a cancer-free individual with a cancerous one could have serious health consequences for both patients. Misdiagnosing an individual with cancer based on a contaminated biopsy sample may result in extreme and unnecessary treatments such as surgery, radiation therapy, or chemotherapy.

Many stages of biopsy analysis, from sample collection to slide preparation, offer an opportunity for contamination. This study grew out of an event in which the Oklahoma State Center for Health Sciences (CHS) forensic department was asked to analyze biopsy tissue suspected of contamination. A patient was diagnosed with uterine cancer on a biopsy. A hysterectomy was performed and no evidence of cancer was found in the uterus. CHS received glass slides of the original uterine biopsy, and extracted DNA from the slides to compare with the patient's DNA. The original uterine biopsy was found to contain DNA from more than one individual, confirming contamination had occurred. The origin of the other DNA profile in the sample could not be determined even after comparing the profile to numerous other samples that had been processed the same day as the original tissue.

This particular contamination event may have resulted from cancerous tissue being accidentally picked up onto a slide with tissue of the cancer-free patient. Most likely, the water bath used to float the paraffin strip after cutting was not thoroughly cleaned between uses, and a piece of cancerous tissue (called a "floater") from a preceding patient may have remained in the water bath. Unknowingly, the technician

may have picked up two types of tissue onto one slide resulting in a slide sample with DNA from two separate individuals.

Resolving contamination events is time-consuming and cumbersome, and quality controls are in place in all hospitals to try to prevent their occurrence. However, these guidelines may not be followed to the letter if a technician is rushed by being over-worked, or not following procedures. Contamination can also occur prior to slide preparation, for example, a contaminant may be embedded in the wax block with the original sample. Embedding tissues in blocks of paraffin wax is customary, and the wax block serves as the preserved source from which sections can be prepared for microscopic examination. Contamination of wax blocks is harder to detect than the odd piece of floating tissue, as it can appear throughout all or multiple slides rather than being only on one slide.[1] Mislabeling samples represents a third important form of contamination.

STR-DNA[a] typing methods can rapidly detect contamination (reflected as DNA profiles from more than one individual), provided adequate amounts of DNA are recovered from the slide or wax blocks. Therefore the need for successful DNA

---

[a] Short tandem repeats (STRs) are highly variable, repeating segments of the genome. The repeats are generally 3 or 4 base pairs in length. STR variability is capitalized on in DNA profiling. The locations of variability are first targeted by primers that are specific for a given genomic sequence and then amplified, or multiplied, using PCR (section 3.4.1 *Quantitative Template Assay Technology*). Certain STRs are common among about 5-20% of the population, so by using 13 genomic locations the individuality of a set of STRs (the genetic profile) can be determined.

extraction from paraffin embedded tissues is critical not only for identification purposes but also for many other applications involving DNA analysis. For example, gene analysis is crucial to cancer research, yet fresh tissue can be difficult to obtain, especially in large quantities and over short time periods, as are often necessary for research. Paraffin-embedded tissue offers a resource of cancerous tissue that is suitable for many purposes if DNA can be successfully extracted.[3] Archived tissues may useful for mutation screening[3] or the detection of pathogens[4]. In addition, DNA analysis is currently being used to direct personalized cancer treatments.

STR-DNA analysis is the primary method used to associate unknown biological material, including that found in a biopsy sample, with an individual. STR profiling involves examining chromosomal "addresses" within the genome, called loci, which vary from person to person. The frequency of each allele at each locus in a given population is known. STR results produced from several of these variable loci are then combined statistically to indicate the likelihood that a biological sample belongs to a specific person. Tissue samples representing biopsies can be genetically analyzed in this manner to indicate the person from whom the sample originated. Moreover, this analysis will reveal if more than one person contributed to the sample—a clear indication of contamination.

Knowing that contamination does occur, a worst-case scenario would include the failure to detect such an event. Successfully extracting DNA from biopsy samples is vital to contamination resolution, but may be prohibited by limitations in DNA quantity, poor PCR amplification, inhibition, or sample degradation. Therefore, the over-arching goal of this study was to critically examine and possibly enhance DNA extraction and

quantitation from biopsy samples. We examined ways to reliably extract and quantify biopsy samples—keeping in mind the limitations involved—regardless of how the tissues were processed. A method to accurately quantify the amount of DNA that should be expected from a biopsy of a given size would greatly assist labs faced with using slides or wax blocks for DNA-based tests. Improving extraction and quantitation methods could help achieve the maximum DNA yield from histological specimens in order to generate an STR profile from this sample type.

Past studies cover DNA extraction methods for tissue embedded in paraffin wax.[5,6,7] Processes for paraffin embedding vary, as do post-paraffin treatments with dyes, fixatives, and stains. While an optimal extraction method for every type of fixation is beyond the scope of this study, the methods most likely to be used will be examined in depth.

Multiple facets of the extraction process will be examined. Tissue samples must be measured, as the amount of starting material generally determines the quantity of resultant DNA. The type of tissue from which DNA is recovered should also be considered. Different tissue sources may yield more or less DNA, owing to structural characteristics of the tissues themselves. Additionally, wax was removed from some samples to observe the effect of wax on DNA yield. Finally, two methods of DNA quantitation were studied, as genetic profiling largely depends on reliable quantitation of unknown samples. The ability of a technique to accurately quantitate yield is as important as the extraction method. DNA recovery was quantified using two extraction methods that both center on the polymerase chain reaction (PCR). PCR has become an

integral part of any analytical method involving nucleic acids. In this study, both Real Time and post-amplification quantitation methods were employed.

Six tissue types were utilized in this study; four main tissues examined were of the colon, liver, lung, and breast, as they represent the most common biopsies performed. Additionally, brain and kidney biopsies were examined, though less intensely. Focusing our study on tissues commonly examined in the pathology laboratory made obtaining adequate numbers of samples easier and also ensured that our results were applicable to the widest population of patients.

Developing the DNA extraction and quantitation process may be beneficial in certain high-cost situations: Loss of life, such as wrongful death, or litigation brought against a hospital may warrant cost-effective DNA profiling. Insight into the extraction process may also benefit current research practice by ensuring that technicians are receiving the maximum amount of yield from a biopsy sample presented to their laboratory.

Chapter II

Review of Literature

An in-depth review of current literature on formalin fixed paraffin embedded (FFPE) tissue was conducted prior to beginning this study. The review focused on foundations of DNA extraction from paraffin, and the application of this technique for possible diagnosis and treatment as well as current extraction methods.

The goal of this literature search was to determine where any gaps in knowledge exist concerning DNA extraction from wax-embedded samples. It is important to place research in the context of what has already been done, so that it will enhance the body of knowledge and further science as a whole. Additionally, the search serves to expose limitations in previous studies to determine if they can be overcome through further experimentation or if they were simply inherent in the research procedure.

2.1. Frequency of Misdiagnoses

One application of STR-DNA analysis that relies on extraction from biopsied tissue is in the detection of DNA foreign to the patient, a result consistent with

contamination. As in the case of uterine biopsy tissue sent to CHS, contamination can lead to an incorrect diagnosis. When an incorrect diagnosis does occur, the results are often profound. If the patient proceeds with a surgical removal of the cancerous region or tumor, the treated area may end up disfigured or non-functioning. In some cases surgery can result in complications leading to additional illnesses or death.[b,8] Preventing unnecessary surgery or chemotherapy treatment is of the greatest concern; implementing genetic examination of suspect samples could assuage this concern.

2.1.1. Resolving Misdiagnoses

When a patient is misdiagnosed with cancer the error is usually discovered at some treatment phase. Post-surgical gross or microscopic analysis can expose the excised mass as healthy or even as a different disease.[2] The patient may have experienced mental anguish and some physical trauma as a result of the ordeal. After a misdiagnosis, the hospital may choose to review their procedures internally.

Recovering from an erroneous diagnosis will be difficult for a hospital. Patients facing cancer and a major surgery have likely taken measures to prepare for death, informed family members of their status, and battled psychological distress. The pain and suffering associated with a cancer pronouncement, compounded by the hospital's desire to avoid negative publicity, could result in a large settlement for a misdiagnosed

[b] While contamination is rare in biopsied tissues, other medical tests such as those requiring cytological specimens experience a higher frequency of contamination. Many of the principles discussed in this study, like areas where contamination may occur, could also be at issue in other diagnostic or therapeutic processes.

patient. Regardless of the outcome, misdiagnoses are never a small incident and are upsetting for all involved.

2.2. DNA Extraction from Formalin Fixed Samples

Before any tissue sample can be genetically analyzed, the DNA must first be released from its cellular environment. In addition to the cellular constraints, DNA in biopsied tissue samples is fixed in 10% formalin—formaldehyde dissolved in water— for the purpose of maintaining the cellular structure so it can be examined microscopically. Formalin-fixed DNA must therefore be 'unfixed' as well as released from the cell. This process of releasing DNA is known as extraction, and can be accomplished in a number of ways. Most extraction methods consist of variations upon the same steps, and finding the optimal process is often accomplished through trial and error.

DNA extraction from formalin fixed samples has been the subject of research for decades, but a universally accepted method has not yet been established. Technicians prefer different methods based on both their personal techniques and the materials they are working with. Some conditions required by a particular extraction method may not be practical for all sample types or workplace environments. Regardless of personal preference, a few established methods do consistently yield better results than others.

2.2.1. Extraction Methods

Paraffin-embedded samples have been subjected to numerous extraction protocols in an attempt to determine optimal conditions. While technicians commonly use commercial kits for simplicity, other extraction techniques can surpass kits' quality in terms of yield of DNA or overall suitability for PCR amplification.[10] We will be using a

kit for this study because it is a proven method, yields reproducible results, and is cost-efficient. Research on paraffin-embedded DNA extraction frequently uses a kit to ensure equal treatment of all samples, while a separate aspect of the process not involving kit reagents is altered as the variable.

Another extraction procedure occasionally used for biopsy samples, phenol-chloroform extraction, takes advantage of the different solubilities of cellular components such as proteins, lipids, and DNA.[c] DNA is successfully separated from other components with this method, however it requires several steps and can be time-consuming. Additionally, the reagents are somewhat more toxic than those in other methods. In one extraction study, Elena Rivero of the Federal University of Santa Catarina, Floriano´polis, SC, Brazil compared a salting-out method with phenol-chloroform extraction and found that the results for both methods were comparable.[4] The salting-out procedure required fewer steps and less-toxic chemicals. Shan-Rong Shi of the University of Southern California Keck School of Medicine examined extraction a varying pH levels, and found that extraction is most efficient at a pH around 9.[6] These techniques and variations should be taken into consideration when optimizing extraction.

---

[c] Phenol-chloroform extraction is a liquid-liquid extraction method commonly used in many forensic and research facilities to isolate DNA. While it can be cumbersome, the technique has persisted due to its ability to yield clean nucleic acid, free of protein or other macromolecules. Initially, lipids and proteins are lysed, and subsequent steps isolate DNA from other cellular material by separation into organic and aqueous phases. This technique can also be used to isolate RNA or DNA.[11]

2.2.1.1. Current Protocol

Currently, Oklahoma State University Center for Health Sciences (CHS) utilizes the TaKaRa DEXPAT kit to extract DNA from tissue samples (Takara Bio Inc, Shiga, Japan). DEXPAT is considered to be a high-quality method of DNA extraction from paraffin wax. This procedure requires a heating step followed by centrifugation, after which the supernatant containing DNA is removed and ready to be amplified. DEXPAT prepares DNA for PCR amplification quickly by eliminating a deparaffinizing step common in other extraction methods. Deparaffinizing is not essential for recovery of high quality DNA, but some find that it improves the yield and/or quality of their extractions.[d] While the quality of DNA may be higher, the disadvantage of removing paraffin is that some tissue is inevitably lost along with the wax, thereby decreasing the final DNA yield. For the purposes of this study, retaining DNA was more important than purifying it, keeping in mind the ultimate goal of generating an STR profile.

2.2.1.1. Sample Size

Selecting the appropriate sample size for analysis is vital as it determines the quantity of DNA that can be potentially recovered. Deciding how much tissue to use can be complex. Large samples may actually yield less DNA suitable for amplification than

---

[d] Deparaffinizing dissolves paraffin wax in order to expose cells for lysis. Typically performed with Xylene, a number of hydrophobic solvents can actually be used. When deparaffinization is incorporated into an extraction method like DEXPAT, sample retention is higher due to eliminating tube transfers and multi-step procedures.[8] Deparaffinizing can also be carried out by heating and cooling the sample so that the paraffin adheres to the tube, although conventional wisdom suggests that tissue in samples with a large amount of paraffin will not be successfully removed.

smaller samples, due to the introduction of PCR inhibitors into the extract. Examining the correlation between sample size and yield may serve as a guide for selecting appropriately sized samples if certain tissue types are consistently more degraded than others, or if DNA yield is affected by age of the sample or presence of inhibitors.

Sections cut from wax blocks are commonly between 5μm and10μm thick. This range is fairly consistent across the field, as smaller sizes may be beyond the lower limit of analysis and larger samples are not always available. The availability of larger slices is limited because biopsy sections more than 10μm thick are not suitable for microscopic analysis. So, as in the case of the aforementioned CHS study, when researchers must work with slices on glass slides they seldom come across samples thicker than 10μm. To recreate these real-world limitations, this study used sections 10μm thick.

2.2.2. Fixation

The fixation process may lead to chemical modifications that bring about base changes and lessen the overall stability of the DNA molecule.[7] Furthermore, prolonged fixation increasingly cross-links proteins and the double helix over time (Figure 1). This may affect development of a DNA profile, as longer sections of the genome may not correctly amplify due to the damage caused by cross-linkages. After analysis, tissues that have been more severely cross-linked show increased fragmentation.[11]

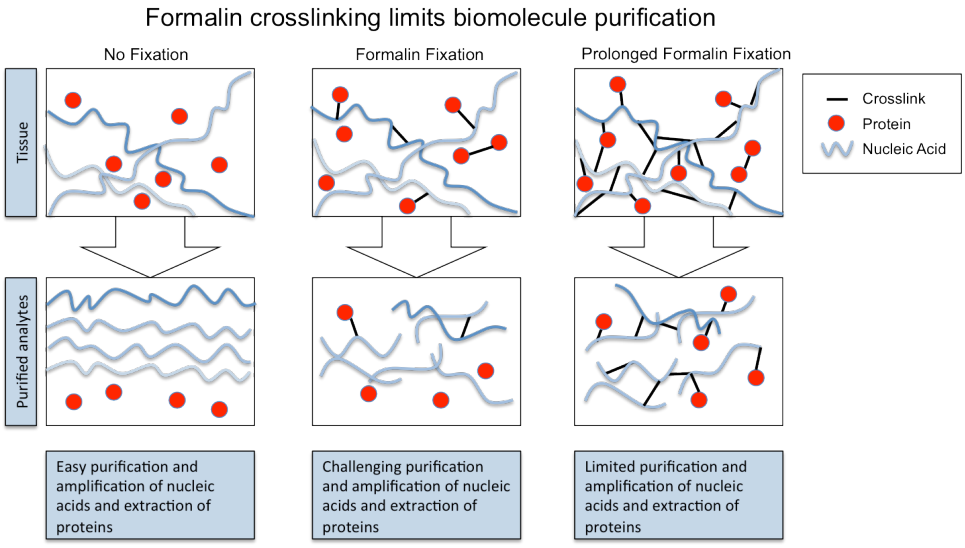


Figure 1. Effects of Fixation on Nucleic Acids
Purification of DNA extracted from paraffin embedded sections is limited by the length of fixation, with longer fixation times leading to a steep increase in cross-links. The result of extensive cross-linking is often evidenced by limitations of purification of the final DNA product.[8]

Multiple studies show that formalin fixation can prevent complete lysis of cells during extraction.[8,12] Some fixative formulas have been shown to minimize some of the degradation that occurs with formalin fixation, but they are conversely much worse for use in histological analysis and also more labor-intensive to produce.[13] Because the primary purpose of tissue fixation is diagnostic pathology, it is unlikely that the switch to more DNA-friendly fixatives will be made unless one is proven to be as effective as formalin. Other fixatives, such as picric acid, destroy DNA entirely, but make tissues extremely receptive to acid dye staining.[14] While the search for the perfect fixative has yet to be identified, fixed tissue samples are still preferable to non-fixed samples, as they are somewhat more protected from degradation during subsequent paraffin embedding.[15]

Further complicating fixation, different tissue types may require different fixation times or procedures. The softer consistency of breast tissue, for example, may necessitate

a longer fixation time than other tissues, which leads to the potential for increased fragmentation of DNA from this source.[11] Variation among tissue treatment supports the evaluation of a wide range of tissue types in this study.

As an aside, fixation may affect mitochondrial DNA (mtDNA) differently than nuclear DNA. Literature suggests that mtDNA may be more resistant to the effects of formalin fixation that nuclear DNA. In future studies, repeating tests with mtDNA could substantially affect results.[13]

2.2.3. Staining

Biopsy samples are usually stained for microscopic analysis, in order to increase the visual contrast of different cellular components. This contrast aides the pathologist in making an appropriate diagnosis. Hematoxylin and eosin (H&E)[e] is the most widely used stain in histology.[16] This acidic dye binds to nuclei, but may also alter the cell in a manner that affects DNA extraction. A study performed by Joanne Simons of the Institute of Environmental and Science Research found that H&E staining had an immediate effect on DNA recovery. However, the DNA yield from ten-week old stained samples was not significantly different than non-stained samples.[18] More research into the effects of staining on DNA yield is needed, but current literature supports the theory

[e] Hematoxylin, oxidized and complexed with aluminum ions, forms hemalum, which colors cellular components—namely nuclei—blue. Eosin Y (bromide oxidized by fluorescein) counterstaining then colors cytoplasm and extracellular proteins shades of red.[17]

that it does negatively affect amplification—and possibly DNA recovery—to some extent.

2.2.4. DNA Inhibition

Inhibition occurs when an external agent interferes with DNA amplification. Inhibitors can be chemicals used when fixing DNA, such as formalin, or chemicals used for analyzing tissue sections, like stains. These agents may block PCR altogether or cause amplification to proceed more slowly by altering or degrading a portion of the genetic material.[19] Inhibitors can reduce the amplification yield by lowering the overall amount of DNA produced, or by binding to DNA template thereby reducing its availability for amplification.[20]

2.2.4.1. Unknown Inhibitors

Like DEXPAT, several DNA extraction methods do not require a deparaffinizing step, yet some researchers have found it to be vital for removing unknown inhibitors.[21] One such study, performed with unstained samples, systematically eliminated known inhibitors, so researchers determined that PCR inhibition may have been cellular in origin rather than attributable to an external source.[7] More research needs to be done to pinpoint what kinds of cellular PCR inhibitors exist.

2.2.5. Degradation

Physically, degradation is the breaking down of the components of the DNA molecule. Depurination, one cause of degradation, is the removal of a purine base (either adenine or guanine) from the sugar "backbone" of the molecule. Depurination can be

induced by heat or acidity and precedes separation of the adjacent phosphodiester[f] bonds.[23] In this form, DNA can no longer be amplified or analyzed.

Degradation is a common source of poor amplification when working with older or otherwise compromised samples. DNA can degrade for a variety of reasons, many of which are due to storage in a hostile environment. While nucleic acids can tolerate a broad range of environmental conditions, exposure to excessive heat, moisture, or acidity will cause DNA to break down into fragments that cannot be successfully analyzed. Because STR analysis requires DNA templates of specific lengths (comprising the panel of different STR loci), any alteration of template length by fragmentation will yield inaccurate results. These degradation effects can be somewhat ameliorated by analyzing shorter genomic segments because they degrade more slowly than longer DNA strands.

The effects of DNA degradation mimic those of low-yield DNA, but can be recognized in analysis by the characteristic downward slope across the electropherogram[24] (Figure 2).

---

[f] A phosphodiester bond is characterized by covalent bonding of a phosphate group in the pentose (five-carbon) sugar of one nucleotide in a DNA strand to the hydroxyl (OH) group in the pentose sugar of an adjacent nucleotide.

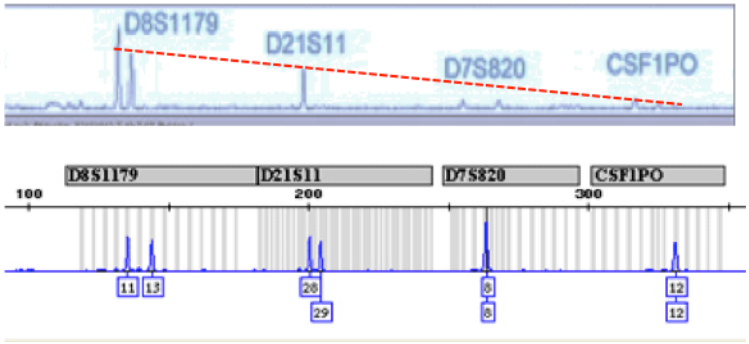


Figure 2. Evidence of DNA degradation
Degradation can be seen when profiling DNA, as in the top electropherogram[17], obtained from a sample of DNA that had been heated in an oven for one month at 56°C. While the peaks on the left side of the figure are high enough to adhere to allele-callings guidelines, they slope downward to the right across the image, indicated by the red line, finally falling below the threshold. The lower electopherogram[24] displays a normal non-degraded profile.

Aside from degradation of FFPE tissue, studies suggest that any extract from these tissues may begin to degrade immediately after extraction. For this reason, extracts should be quantitated as rapidly as possible. Takara Bio claims that DEXPAT extracts can be stored for "up to 3 months at 4°C and up to 1 year at -20°C". [25] So, while it was not a main focus in this study, time sensitivity of the extracts was taken into consideration in this study in order to prevent extract degradation from altering results.

Chapter III

Methodology

3.1. Overview

As previously stated, one goal of this study was to assess methods of reliably extracting DNA from wax embedded biopsy samples. Factors that may inhibit or reduce DNA yield were a main focus, and included: the size of the biopsy sample; the effect of paraffin wax on extraction and/or PCR amplification; tissue type, and PCR inhibition. One specific question addressed in this study was whether or not a relationship exists between sample size and the amount of DNA recovered from that sample. While seemingly an obvious correlation, the two may not relate as expected. Examining tissue size vs. DNA yield also opened the possibility of determining an average amount of DNA recovered per cubic micron of sample material.

3.2. Sample Preparation

Biopsied tissues are commonly fixed in formalin and embedded in wax for preservation and microscopic examination.[26] Formalin cross-links the cell structures so that they do not change or degrade prior to microscopic analysis.[26] Most hospitals use a 10% buffered formalin solution for fixation. However, the length of fixation times vary widely[10], while the penetration rate of formalin through the tissue is fairly constant at about 1mm per hour[26]. After fixation, the sample is dehydrated so that all of the water in the tissue is replaced with hardened wax. Dehydration is generally performed with ethanol; the dehydrating agent is gradually replaced with paraffin in several successive liquid-changing steps, each of an increasing concentration.

Samples for this project were obtained from the OSU Medical Center, already formalin-fixed and embedded in paraffin wax blocks. These samples had been stored past the length of time required by patient record regulations (10 years) and could therefore be discarded by the hospital. Tissue samples from individuals diagnosed with common cancer types were selected—primarily lung, breast, colon, and liver—so that the study would apply to as broad a patient population as possible.

In total, twenty-one wax blocks from the OSU Medical Center were selected (Table 1). For purposes of this research, the tissue needed to resemble as closely as possible actual conditions in which a lab might receive a biopsy sample. The blocks were sent to Regional Medical Laboratories (RML) to be cut into 15 slices at 10μm thick, put on positive charge glass slides, and air-dried. Each section was stored at room

temperature until DNA extraction and quantitation. DNA extracts were subsequently stored at 4°C.

Table 1 Tissue Samples

SAMPLE TYPE	SEX	AGE	DIAGNOSIS
COLON	M	56	Hx colonic adenocarcinoma. Moderately differentiated: Focal mucinous features.
COLON	M	65	Ulcerated circumferential colon mass. Invasive moderately differentiated adenocarcinoma.
COLON	M	66	Moderately differentiated adenocarcinoma
COLON	F	80	Invasive moderately differentiated adenocarcinoma
COLON	F	81	Invasive adenocarcinoma, moderately differentiated
COLON			Transmural chronic inflammation: suggestive of Crohn's disease
BREAST	F	54	No distinct mass, Fibrocystic change with stromal fibrosis and adenosis. Defer final diag pending additional tissue.
BREAST	F	59	Infiltrating ductal carcinoma, moderately differentiated. DCIS intermediate grade.
BREAST	F	63	Infiltrating ductal carcinoma, poorly differentiated focal DCIS, high grade comedonecrosis
BREAST	M	32	Gynecomastia, no malignancy
BREAST	F	83	Infiltrating lobular carcinoma, solid and classical types
LUNG	F	72	Small cell carcinoma
LUNG	M	61	Small cell carcinoma
LUNG	M	72	Non small cell carcinoma
LUNG	F	75	Small cell carcinoma
LIVER			Small cell carcinoma
LIVER			Poorly differentiated malignant neoplasm; diagnosis favors adenocarcinoma
LIVER			Poorly differentiated carcinoma consistent with metastatic renal cell carcinoma
LIVER			Necrotic inflammation
BRAIN			Malignant
KIDNEY			Papillary Transitional Cell carcinoma

This table lists each biopsy sample obtained from the OSU Medical Center, along with all of the information known about that sample. While many biopsies contained information on the gender and age of the patient, several did not, yet the information was retained out of interest. The diagnoses made based on these biopsies were primarily malignant.

3.3. Takara DEXPAT Extraction

The TaKaRa DEXPAT DNA extraction kit is distributed by the Japanese company, Takara Bio Inc (Shiga, Japan), and is specifically formulated to optimize DNA extraction from tissues that have been formalin-fixed and embedded in paraffin wax. Wax complicates biological tissue extraction, as paraffin may inhibit the effectiveness of some extraction reagents. Additionally, if all of the wax is not removed, traces left

behind can interfere with DNA amplification and STR analysis. An extraction process like DEXPAT that is designed to work with paraffin wax claims to enhance final DNA yield and quality.

The procedure for DEXPAT extraction is very straightforward. DEXPAT extracts DNA in one step, meaning there is no need for an initial wax removal step. Instead, DNA is extracted from both the wax and the cell simultaneously. Five tissue sections of the desired size (in this case 10μm thick) were placed in a 1.5ml microfuge tube. Next, twenty drops of DEXPAT extraction reagent (about 0.5ml) were added to each tube. These tubes were heated at 100°C for 10 minutes, followed by centrifugation at 12,000 rpm and 4°C for 10 minutes. Centrifugation separates the DNA extract from melted paraffin, cellular components, and the DEXPAT reagent. After centrifugation, a top layer of paraffin, hardened by exposure to low temperatures, was punctured with a pipette tip and the supernatant below transferred to a clean tube. The precipitate was discarded.

A process to further purify the DNA and capture it in a small volume followed the extraction procedure. Using the Zymo Genomic Clean and Concentrator^{TM} kit (Zymo Research, Orange, CA), the extract was first mixed with the kit's DNA Binding Buffer, which facilitates the binding of DNA to the Zymo-Spin^{TM} Column, which contains silica (Figure 3). Binding buffer volume was approximately twice the extract volume, as dictated by Zymo kit protocol. The extract was vortexed well with the binding buffer and loaded onto the silica spin column, located in a 1.5mL microfuge tube. The column was then centrifuged at 12,000 rpm for one minute, during which DNA was bound to silica

contained within the spin column, while impurities flowed through into the microfuge tube.

Figure 3. Spin Column
The Zymo-Spin™ Column collects DNA on a silica membrane while cellular "junk" flows through, allowing for purification and concentration of DNA. [28]

After the binding step, the column was washed twice with the Zymo DNA Wash Buffer to remove any residual DNA contaminants. DNA was eluted from the silica column with 30µl of TE-4 at 65°C (10µM Tris-Cl, pH 8.0 with 0.1µM EDTA).[g] Storing DNA in TE-4 rather than water prevents some DNA damage from long-term storage. Elution was performed in two steps: 15µl of hot (65°C) TE-4 was added to the Zymo-Spin™ Column, to elute the DNA during a one-minute incubation at 65°C. The column was then centrifuged for 1 minute at 12,000 rpm, and the process was repeated for a final

[g] Tris maintains the pH of a solution, while EDTA prevents DNA degradation by chelating metal ions like Mg^{2+} that may otherwise catalyze the hydrolysis of DNA.[29,30]

volume of 30µl. The extract was immediately quantitated (section 3.4 *Quantitation*) then stored at 4°C.

Using DEXPAT decreases the amount of time necessary for a typical extraction process by eliminating an initial deparaffinizing step, which typically includes a Xylene/alcohol washing process and also requires allowing time for the tissue to dry after wax is removed. Traditional extraction is done with phenol and chloroform in a multi-step purification and centrifugation process (Figure 4).

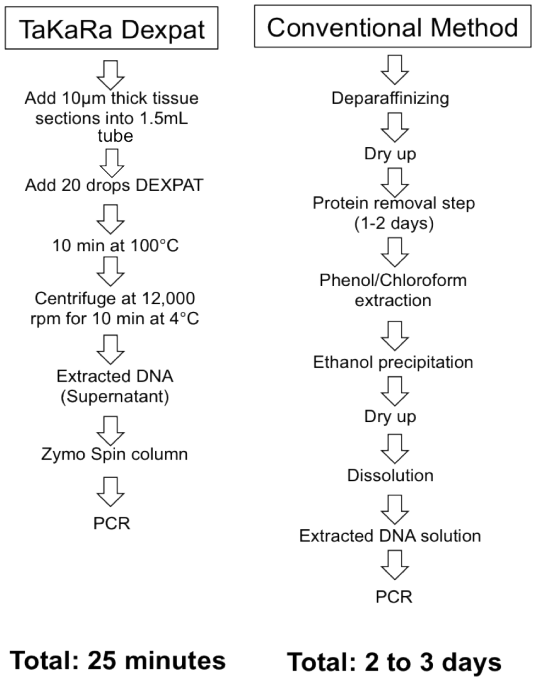


Figure 4. Extraction Methods
Comparison of DEXPAT extraction with conventional extraction methods. DEXPAT eliminates the preliminary deparaffinizing step.

3.4. Quantitation

Following extraction, DNA recovered from the biopsy tissues immediately underwent one of two quantitation procedures to estimate the total amount of DNA

present. These two procedures are fundamentally different; yet seek to accomplish the same goal.

The first method, the Quantitative Template Assay Technology (Q-TAT), is an end-point quantitative technique developed at CHS. [31,32] End-point methods quantitate DNA in a sample after is has undergone PCR amplification. Q-TAT allows a scientist to determine the amount of DNA in an unknown sample by comparing the fluorescence of several amplicons produced by the sample to that produced by a DNA standard. Q-TAT primers amplify the amelogenin (or AMEL) locus on the X and Y chromosome, which means they can also act in a sex-typing function. Sex typing can be useful as a preliminary examination step when two potential donors for a suspected contaminated sample are different genders. Furthermore, Q-TAT results can predict the state of degradation of DNA in a sample. [34,33]

The second quantitative method used in this study was Real Time PCR using the Quantifiler kit from Applied Biosystems (Foster City, CA), also called qPCR to indicate its quantitative function. While Q-TAT requires a post-amplification capillary electrophoresis step to estimate the quantity of DNA present in a sample, a Real Time PCR system determines DNA quantity as the reaction progresses; qPCR is therefore a somewhat faster technique. Real Time PCR was performed with the Quantifiler© Human DNA Quantification Kit from Applied Biosystems (Foster City, CA).

Comparing these two methods may offer insight into the nature of DNA recovered from FFPE tissues, as both techniques offer unique advantages. The method of detecting inhibition inherent in Q-TAT can suggest at a glance that a PCR reaction is

inhibited, while qPCR is known for its accuracy and tracks the kinetics of a PCR reaction as it progresses.[33]

3.4.1. Quantitative Template Assay Technology (Q-TAT)

Setting up a reaction for QTAT may not differ noticeably from a Real Time reaction at first glance, however Q-TAT differs from Real Time PCR in a number of ways, the first of which will be discussed in this section. While both methods rely on the same basic components common to all PCR reactions, primers used in Q-TAT select for different genomic target sequences than those in qPCR. Additionally, these primers have been tagged with the fluorescent dye FAM, which allows a genetic analyzer to determine the amount of DNA present in a sample based upon the amount of fluorescence a PCR product emits.

Aside from the two AMEL X and Y primers, Q-TAT includes two additional primers designed to specifically quantify male or female nuclear DNA. On the Y chromosome, the SRY gene is targeted to identify male DNA, while the HPRT gene (labeled HP in Q-TAT electropherograms) on the X chromosome can estimate the quantity of female DNA in a mixture of male and female DNA. Also in the primer mix are primers targeting two regions of mitochondrial DNA (mtDNA), producing NED-labeled amplicons of 287 and 97 bp.[34] The ample amount of mtDNA in the cell as compared to nuclear DNA (nDNA) may offer Q-TAT a unique quantitative advantage over methods analyzing strictly nDNA.

In addition to human-directed primers, a pRL plasmid and primers are added to the Q-TAT PCR reaction to detect inhibition. The pRL plasmid harbors a gene encoding

luciferase found in *Renila rentiformis*. At 200 base pairs, the plasmid is sized closely to the AMEL X and Y genes, yet just different enough to be distinguished.[36] If a reaction is inhibited, pRL fluorescence will be reduced in the samples, which is easily visualized during analysis. If a DNA sample fails to amplify while pRL can be seen at the expected amount, then the scientist can deduce that there is no DNA recovered or that the DNA is degraded, rather than attributing the failure to PCR inhibition. Knowledge of potential inhibition is particularly useful in this study because paraffin embedding may contribute to inhibition of amplification.

Q-TAT reactions for extracted DNA samples of unknown quantity were run alongside a serial dilution of specific known quantities. The relative fluorescent units (RFUs) of the known quantities are used to form a standard curve (Figure 5).

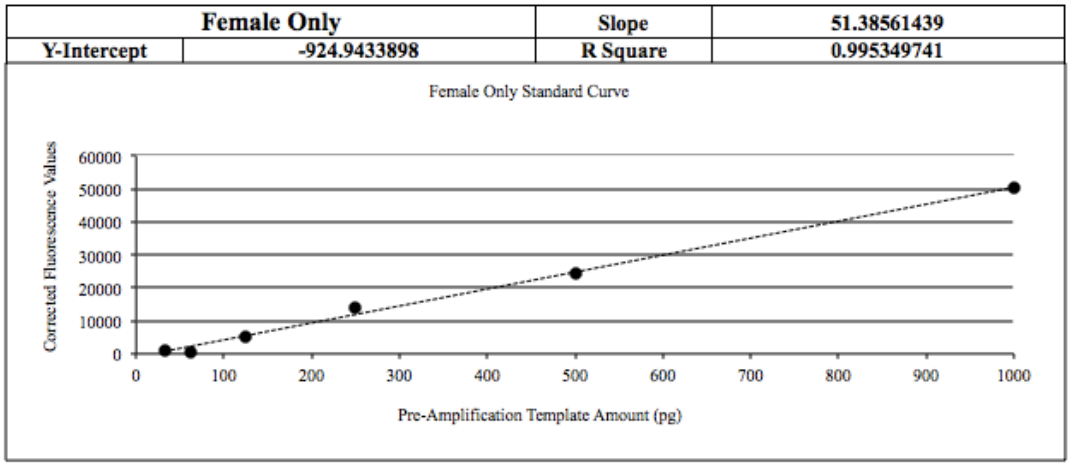


Figure 5: HP Standard Curve
The serially diluted known DNA quantities are used to generate a standard curve. A high R^2 value indicates the sample points closely adhere to the plot line, where a value of 1.0 is perfect adherence. R^2 values for HP were superior to those for AMEL and SRY for most Q-TAT amplifications.

Fluorescence in extracted unknowns was compared to the standard curve, and an estimation was made as to their respective DNA quantities. Running the standards with every set of unknown samples helps to minimize variation between standards and unknowns due to mechanical errors. The standard dilutions were prepared from stock male DNA, previously quantitated at 100ng/λ. This stock was diluted 1:100 with DI water to make the 1000pg/μL standard, and then 7.5μL were added to 7.5μL DI water in a 0.2mL PCR reaction tube, to make the other 2-fold serial dilution of DNA for the standard curve. Serial dilutions yielding 500ng/μL, 250ng/μL, 125ng/μL, 62.5ng/μL, and 31.25ng/μL were created with a 0ng/μL negative control. 1μL of each serially diluted sample was added to a new PCR reaction tube and amplified in the Q-TAT assay.

Each unknown or standard DNA sample was amplified in a PCR tube with 1μL of extracted DNA and 11.5μL of a master mix consisting of 1.75μL of water, 1.25μL10x primers, 0.5-1pg of pRL DNA in a 1μL volume, and 7.5μL Hot Start GoTaq amplification mix (Promega Corp, Madison, WI). This master mix was prepared, then 11.5μL aliquots were dispensed to the DNA samples. The Taq polymerase was a hot-start variety (Promega Corp, Madison, WI) to reduce the potential for non-specific primer annealing, which can occur at lower temperatures.[36]

After each sample was prepared it was vortexed. If liquid adhered to the tube wall as a result of vortexing, the sample was spun in a microfuge. Tubes were then placed in an ABI 9700 thermocycler (Applied Biosystems, Foster City, CA). Cycling conditions for the Q-TAT assay are summarized below in Figure 6.

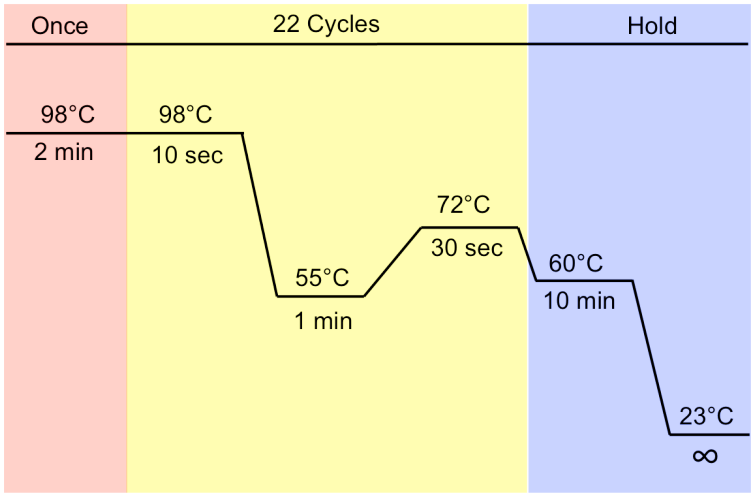


Figure 6: **Q-TAT PCR Amplification Parameters**
Q-TAT begins with a heating step at 98°C for 2 minutes to activate the Taq polymerase. Then the actual cycling begins with a 10 second step at 98°C, followed by a temperature drop to 55°C for 1 minute. Because DNA primers are highly concentrated in the solution, they bind to the template DNA as it is cooled. The third step in the cycle is at 72°C for 30 seconds, which allows for extension of the primers by addition of dNTPs. This tri-step cycle is repeated 22 times. After cycle completion, the thermocycler holds the DNA samples at 60°C for 10 minutes, then 23°C indefinitely.

3.4.1.1. Capillary Electrophoresis

PCR products, or amplicons, were prepared for analysis on the ABI 310 genetic analyzer (Applied Biosystems Inc, Foster City, CA) by adding 1μl of the amplicon to 24μl of a 1:100 Liz/Formamide Hi Di solution (GeneScan™ 500 LIZ ™ Size Standard by Applied Biosystems, Foster, CA). Samples were mixed in 750μl tubes and placed in an ABI 310 sample tray.

Once the sample tray was prepared, samples were analyzed on an Applied Biosystems 310 Genetic Analyzer. The ABI 310 separates amplicons in each sample

tube electrophoretically, and captures the fluorescence (RFU) of the amplicons. The GeneMapper ID software (ver. 3.2) was used to analyze the ABI 310 results. By reading the RFU of an individual amplicon in a sample and comparing it to the standard curve, the amount of DNA present in the original DNA extract can be determined.

GeneMapper ID labels each amplicon in a Q-TAT result with peak height and peak area fluorescence. We chose to quantify based upon peak area, as it revealed more consistent and accurate results (higher R^2 values) than peak height. Values for each amplicon in each sample in the standard curve were entered into an excel spreadsheet (Appendix A). This spreadsheet also allowed for the input of unknown RFU values and compared the RFU of unknowns to the standard curve, producing an estimated DNA quantity. In this way, quantities were assigned to unknowns for the AMEL X and Y, SRY, and HP loci

3.4.2. Real Time

This real time qPCR kit (Quantifiler Human DNA Quantification Kit, Applied Biosystems Inc, Foster City, CA) includes a proprietary primer mix, stock DNA at 200ng/μL, and a PCR reaction mix containing dNTPs. Similarly to Q-TAT, real time amplification requires the generation of a standard curve from known quantities of DNA to estimate the DNA quantity in unknowns. This curve is prepared in much the same manner as the Q-TAT curve, but the reagent concentrations differ slightly.

10μL of stock DNA from the Quantifiler kit is added to 30μL of TE-4 to make a 50ng/μL sample (a 4X dilution factor). 10μL of this pipette-mixed solution is added to 20μL of TE-4, resulting in a 16.7ng/μL sample (a 3X dilution factor). This serial dilution

then continues at 3X dilutions, with 10uL of the 16.7ng/μL added to 20μL TE-4, and so on, yielding known samples of 5.56ng/μL, 1.85ng/μL, 0.62ng/μL, 0.21ng/μL, 0.068ng/μL, and 0.023ng/μL. Finally, 1.2μL of each serially diluted sample is added to a separate well in a 0.2ml Microamp^{TM} Optical 8-tube Strip. Likewise, 1.2μl of each unknown extract is added into wells on a separate strip.

The master mix for qPCR was prepared in such a way that the final reaction volume totals 15μL. This called for 7.5μL reaction mix and 6.3μL primers to be added to a master mix for each sample, plus about 10% extra to allow for error. This is a deviation from the kit instructions, which recommend 10.5μL Quantifiler Human Primer Mix, 12.5μL Quantifiler PCR Mix, and 2μL sample for a total reaction volume of 25μL. Using reduced reagent amounts can lead to poor results, which was found to be the case in early experimental runs. These runs were conducted at total reaction volume of 10μL; after several runs produced poor results the reaction volume was increased to 15μL, which proved small enough to reduce reagent use thereby preventing unnecessary expenses, yet large enough to produce consistent results. Only runs at 15μL were reported.

After 13.8μL of the master mix was added to each sample, the tubes were capped with a Microamp^{TM} Optical 8-Cap Strip, which snaps into the top of each tube in the strip. Each 8-tube strip was vortexed and centrifuged before amplification (Figure 7).

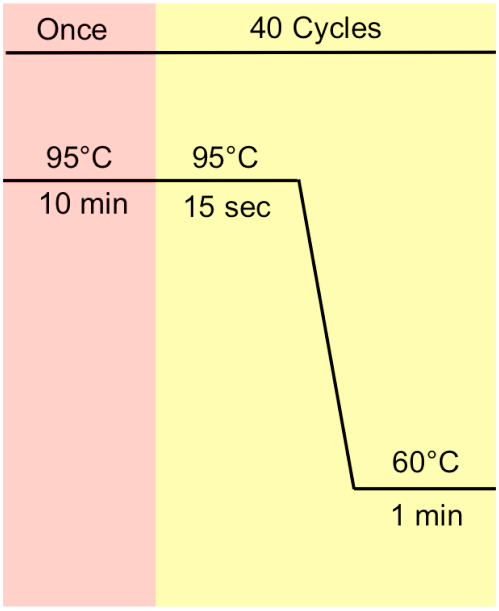


Figure 7 Real Time qPCR Amplification Parameters
Real time begins with a heating step at 95°C for 10 minutes. Then the actual cycling begins with a 15 second step at 95°C, followed by a temperature drop to 60°C for 1 minute. Due to the short amplicons it produces, real time qPCR can combine annealing and extension into this one 60°C step and therefore does not need to include a mid-range temperature like that seen in Q-TAT (Figure 6) into the cycling.

3.5. Experimental Control

The standard curve samples serve as experimental controls, and were amplified alongside all unknown DNA samples. The zero value in the Q-TAT known samples served as a negative control, and each of the other six samples served as positive controls. Negative controls act to ensure that the procedure has not been contaminated by any outside sources, such as experimenter DNA. If the negative control shows evidence of biological material, this indicates that other samples in the same run may also be contaminated. Over the course of this research, no negative controls suggested contamination. A positive control acts as a check on the reagents and processes of a

reaction. It contains a known sample or value that, if incorrectly processed, will indicate that other samples may not yield accurate values.

Chapter IV

Results

4.1. Measurement

For each sample the total tissue area, as well as the area of the surrounding wax, was measured. Accurate measurement of tissue area was difficult for several reasons: Most tissues were of an irregular shape, requiring a meticulous analysis of area, or regular shape that allowed for a general length by width measurement. In this study, a general measurement was perceived to be the most efficient measurement method for all samples given time constraints. Only a marginal benefit was expected with more accurate, yet laborious, measurements. Aside from tissue shape, tissue transparency also complicated measurement by making the discernment between tissue and wax sometimes almost imperceptible. Lastly, a few biopsies—originating from liver or colon tissues—possessed a spotted or speckled appearance, rendering even fastidious measurements imprecise because most spots were smaller than the lower limit of the measuring tool used.

Several alternatives to measuring with a ruler were proposed, but none were deemed to be superior. One alternative included tracing an outline of the tissue on paper, then cutting out the outline and weighing it. However, this would not remedy the transparent tissue problem. Another alternative was to use a computer program to measure scanned images of the tissue, but such a program was not readily available nor allowed for in the budget.

Again, due to a limited benefit from more exact measurements, the original ruler method was perpetuated. This decision does leave room for improvement in future research. The ruler method was consistently applied, thus providing some degree of reliable comparison between extracts. Measurements were as follows:

Table 2 Slice Measurements

Sample Type	ID	Block Surface (cm)	Section Dimensions in cm (all 0.001cm thick) (Replicate slices used for DNA extraction)					Tissue Area (cm^3)	Excess Wax (cm^3)
Colon	3968	3.0 X2.2	2.4 X1.6	2.5 X1.2	2.5 X1.5	2.5 X1.5	2.2 X1.7	0.01808	0.01492
Colon	3968	3.0 X2.1	2.7 X1.4	2.3 X1.5	2.5 X1.5	2.4 X1.3	2.3 X1.5	0.01755	0.01395
Colon	5040	1.3 X1.5	0.2 X0.2	0.2 X0.25	0.2 X0.2	0.2 X0.2	0.2 X0.2	0.00021	0.00954
Colon	7464	1.2 X1.25	0.8 X0.5	0.7 X0.8	0.8 X1.0	0.8 X0.8	0.7 X0.8	0.00296	0.00454
Colon	7464	1.2 X1.2	0.5 X0.7	0.7 X0.7	0.7 X0.7	0.6 X0.6	0.6 X0.7	0.00211	0.00509
Colon	6294	3.0 X2.1	1.5 X1.0	1.5 X1.0	1.5 X1.0	1.5 X1.3	1.2 X1.0	0.00765	0.02385
Colon	6294	2.7 X2.2	1.9 X1.2	1.5 X1.2	1.5 X1.3	1.6 X1.0	1.5 X1.0	0.00913	0.02057
Colon	7057	2.7 X2.2	1.5 X1.2	1.7 X1.1	1.7 X1.2	1.5 X1.2	1.6 X1.1	0.00927	0.02043
Colon	7057	2.7 X2.2	1.4 X1.4	2.0 X1.4	1.7 X1.3	1.5 X1.5	1.4 X1.3	0.00905	0.02065
Breast	2163	3.5 X2.35	2.1 X1.7	2.2 X1.7	2.2 X1.7	2.2 X1.7	2.2 X1.7	0.01853	0.02260
Breast	2163	3.3 X2.2	2.2 X1.7	2.2 X1.8	2.3 X1.8	2.2 X1.8	2.3 X1.8	0.01994	0.01636
Breast	2163	3.2 X2.2	2.2 X2.1	2.2 X2.1	2.1 X2.0	2.0 X2.2	2.1 X2.0	0.02204	0.23426
Breast	2163	3.2 X2.2	2.3 X2.0	2.3 X2.1	2.3 X2.0	2.3 X2.1	2.2 X2.0	0.02326	0.23304
Breast	2445	2.8 X2.2	2.7 X1.3	2.8 X1.3	2.7 X1.4	2.7 X1.5	2.7 X1.4	0.01876	0.01204
Breast	2445	2.7 X2.4	2.7 X2.1	2.5 X2.0	2.5 X2.0	2.6 X1.9	1.9 X2.2	0.02236	0.01004
Breast	2445	2.9 X2.3	2.6 X2.0	2.6 X1.8	2.5 X2.0	2.5 X1.8	2.5 X1.7	0.02363	0.00972
Breast	2445	2.8 X2.3	2.5 X1.5	2.5 X1.8	2.5 X1.6	2.5 X1.8	2.6 X1.8	0.02143	0.01077
Breast	2445	3.0 X2.3	2.8 X1.5	2.8 X1.5	2.9 X1.4	2.8 X1.5	2.9 X1.5	0.02101	0.01349
Breast	5511	3.6 X2.2	2.3 X1.6	2.3 X1.7	2.1 X1.6	2.4 X1.7	2.4 X1.7	0.01911	0.02049

Sample Type	ID	Block Surface (cm)	Section Dimensions in cm (all 0.001cm thick) (Replicate slices used for DNA extraction)					Total Tissue Area (cm^3)	Excess Wax (cm^3)
Breast	5511	3.4 X2.3	2.5 X1.2	2.0 X1.5	2.4 X1.4	2.5 X1.4	2.5 X1.0	0.01536	0.02374
Breast	5511	3.3 X2.0	2.2 X1.6	2.3 X1.7	2.3 X1.6	2.3 X1.7	2.3 X1.6	0.0187	0.0143
Breast	6811	2.4 X2.2	2.4 X1.3	2.2 X1.3	2.3 X1.3	2.2 X1.3	2.2 X1.4	0.01491	0.01149
Breast	6811	2.8 X2.4	2.5 X1.4	2.4 X1.4	2.4 X1.3	2.4 X1.2	2.4 X1.3	0.01598	0.01762
Lung	243	1.1 X1.1	0.6 X0.6	0.6 X0.6	0.6 X0.7	0.6 X0.7	0.7 X0.7	0.00205	0.004
Lung	243	1.2 X1.1	0.6 X0.6	0.5 X0.6	0.7 X0.6	0.5 X0.7	0.5 X0.7	0.00178	0.00482
Lung	5266	3.5 X2.3	2.6 X1.2	2.7 X1.5	2.7 X1.3	2.5 X1.2	2.7 X1.3	0.01719	0.02306
Lung	5266	3.1 X2.9	2.5 X1.2	2.6 X1.3	2.4 X1.2	2.6 X1.3	2.7 X1.2	0.01588	0.02907
Lung	5266	3.1 X2.9	2.6 X1.2	2.6 X1.4	2.6 X1.4	2.6 X1.2	2.5 X1.3	0.01677	0.02818
Lung	5266	3.5 X2.2	2.8 X1.5	2.7 X1.5	2.6 X1.5	2.6 X1.2	2.6 X1.2	0.01839	0.02011
Lung	6829	2.8 X2.2	1.9 X1.8	1.8 X1.8	1.8 X1.8	1.9 X1.8	1.9 X1.8	0.01674	0.01406
Lung	6829	2.7 X2.4	1.8 X1.8	1.8 X1.8	1.8 X1.8	1.8 X1.8	1.8 X1.8	0.0162	0.0162
Lung	6849	1.2 X1.1	0.4 X0.4	0.4 X0.4	0.4 X0.5	0.4 X0.5	0.4 X0.5	0.00092	0.00568
Lung	6849	1.2 X1.2	0.7 X0.5	0.5 X0.6	0.5 X0.6	0.4 X0.6	0.6 X0.5	0.00149	0.00571
Liver	468	2.7 X2.2	2.7 X2.2	2.7 X2.2	2.7 X2.2	2.7 X2.2	2.7 X2.2	0.0297	0
Liver	468	2.8 X2.2	2.8 X2.2	2.8 X2.2	2.8 X2.2	2.8 X2.2	2.8 X2.2	0.0308	0
Liver	4681	0.8 X1.2	0.8 X0.5	0.8 X0.4	0.4 X0.7	0.8 X0.5	0.3 X0.7	0.00161	0.00319
Liver	4681	1.2 X0.9	0.6 X0.2	0.7 X0.2	0.4 X0.4	0.3 X0.3	0.7 X0.3	0.00072	0.00408
Liver	4792	2.2 X2.0	1.5 X1.2	1.4 X1.3				0.00362	0.00518
Liver	4792	2.2 X2.0	1.2 X1.3	1.4 X1.5				0.00226	0.00654
Liver	4792	2.2 X2.0	1.2 X1.4	1.2 X1.2				0.00312	0.00568
Liver	4792	2.2 X2.0	1.3 X1.1	1.1 X1.2				0.00275	0.00605
Liver	5178	2.3 X2.4	1.8 X1.4	1.9 X1.4	1.8 X1.4			0.0077	0.00886
Liver	5178	2.3 X2.5	2.0 X1.5	1.8 X1.5	1.7 X1.2			0.00774	0.00882
Liver	5178	2.3 X2.1	1.7 X1.1	1.9 X1.1	1.2 X1.3	1.9 X1.1	1.8 X1.2	0.00977	0.01438
Brain	147	1.3 X1.3	0.8 X0.4	0.6 X0.4	0.6 X0.4	0.7 X0.4	0.7 X0.3	0.00129	0.00716
Brain	147	1.3 X1.3	0.7 X0.4	0.7 X0.3	0.7 X0.4	0.7 X0.3	0.7 X0.4	0.00126	0.00719
Kidney	39	1.3 X1.0	0.7 X0.5	0.6 X0.3	0.7 X0.7	0.8 X0.5	0.4 X0.2	0.0015	0.005
Kidney	39	1.2 X1.0	0.7 X0.5	0.9 X0.5	0.4 X0.6	0.7 X0.4	0.5 X0.3	0.00147	0.00453

"Block surface" includes all wax on the slide, representing the surface of the block from which the section was taken. The "section dimensions" refer to the area of tissue sections alone, not including wax. Five replicate slices were used for most extracts and each tissue section is represented in the table, while an average block surface area is listed due to very little variation. Samples 3968 and 7464 were classified as "spotted", meaning tissue was not consistently solid throughout the sample.

4.2. Q-TAT Quantities

Based on the standard curve generated with each run, unknown samples were quantitated with Q-TAT at the AMEL X and Y, SRY, and HP loci. Results for HP were consistently superior to other loci, and are thus the only results reported. One explanation for getting results with HP rather than AMEL may be that the DNA is degraded. This is expected given the fixation sections undergo as part of their processing. Furthermore, the acidic H&E stain is commonly applied to tissues (although not the ones used in this study) offering a further opportunity for degradation. These reasons will be discussed further in section 5.1 *Q-TAT*. HP results were as follows:

Table 1 DNA Quantities for Q-TAT (HP) and Real Time qPCR

Tissue Type	ID	DNA/µL of extract*	Total DNA (pg) Q-TAT	pg DNA/mm^3 tissue Q-TAT	Total DNA (pg) qPCR	pg DNA/ mm^3 tissue qPCR
COLON	3968	30.333	910	167.77	104.80	579.65
COLON	3968	38.067	1142	216.90	80.80	460.40
COLON	5040	IND	<32	IND	1.14	544.76
COLON	7464	10.800	324	364.86	3.12	105.27
COLON	7464	48.800	1464	2312.80	41.60	1971.56
COLON	6294	16.033	481	209.59	51.20	669.28
COLON	6294	23.600	708	258.49	29.88	327.27
COLON	7057	76.417	2292.5	824.34	48.00	517.80
COLON	7057	123.550	3706.5	1365.04	46.40	512.65
BREAST	2163	21.367	641	107.15	17.28	86.66
BREAST	2163	34.833	1045	187.98	50.80	274.15
BREAST	2163	10.167	305	46.13	57.60	261.34
BREAST	2163	34.400	1,032	147.89	45.20	194.33
BREAST	2445	5.967	179	31.81	21.48	114.50
BREAST	2445	4.583	137.5	20.50	4.88	21.82
BREAST	2445	16.917	507.5	71.59	16.12	68.22
BREAST	2445	66.767	2003	311.56	50.40	235.18
BREAST	2445	9.933	298	47.28	46.00	218.94
BREAST	5511	1.500	45	7.85	15.80	82.68
BREAST	5511	IND	<32	IND	6.04	39.32

Tissue Type	ID	DNA/µL of extract*	Total DNA (pg) Q-TAT	pg DNA/mm^3 tissue Q-TAT	Total DNA (pg) qPCR	pg DNA/ mm^3 tissue qPCR
BREAST	5511	3.250	97.5	17.33	10.60	56.53
BREAST	6811	24.233	727	162.53	22.40	150.23
BREAST	6811	237.417	7122.5	1485.71	107.60	673.34
LUNG	243	9.367	281	456.91	10.48	511.22
LUNG	243	6.183	185.5	347.38	9.80	550.56
LUNG	5266	189.100	5673	1190.81	56.00	352.64
LUNG	5266	258.733	7762	1542.83	94.00	560.52
LUNG	5266	56.700	1,701	308.32	156.80	852.64
LUNG	5266	50.700	1521	294.94	114.40	665.50
LUNG	6829	34.167	1025	210.91	114.80	708.64
LUNG	6829	40.400	1212	241.34	188.80	1127.84
LUNG	6849	IND	<32	IND	10.44	1134.78
LUNG	6849	23.900	717	1604.03	14.12	947.65
LIVER	468	6.067	182	20.43	12.56	42.29
LIVER	468	9.017	270.5	29.27	8.04	26.10
LIVER	4681	4.000	120	248.45	2.54	158.01
LIVER	4681	4.300	129	597.22	0.24	32.78
LIVER	4792	3.800	114	104.97	4.28	118.23
LIVER	4792	1.633	49	72.40	1.48	65.43
LIVER	4792	2.067	62	66.24	0.75	24.10
LIVER	4792	1.933	58	70.30	2.10	76.22
LIVER	5178	6.400	192	69.26	22.04	238.53
LIVER	5178	5.433	163	70.56	33.32	432.73
LIVER	5178	30.867	926	315.93	62.40	638.69
BRAIN	147	10.400	312	806.20	3.52	272.87
BRAIN	147	8.033	241	637.57	12.04	955.56
KIDNEY	39	4.567	137	304.44	1.59	105.87
KIDNEY	39	5.033	151	342.40	3.26	221.50

This table lists HP values estimated by the Q-TAT standard curve in column "Total DNA (pg) Q-TAT". Original DNA quantities were calculated using the equation $C_1V_1=C_2V_2$. Where "<32"pg is reported in the HP column, this means that the quantity of unknown DNA fell below the lower limit of detection for the ABI 310, and the quantity of original DNA is therefore indeterminable, denoted "IND" in the table. 1uL of the extract was amplified in the Q-TAT reaction, and 1.2 uL in Real Time qPCR. Extract volume for all samples was 30uL. A normalization of the data is presented in the column "pg DNA/mm^3 tissue Q-TAT". The normalization is replicated for Real Time quantities in the far right column.

* Based upon RFU in HP amplicon

HP results are reported preferentially over SRY and AMEL. When SRY results were obtained, the values appear to trend similarly to HP values. For example, where HP results were indeterminable, such as for male sample 5511, SRY results were likewise indeterminable. SRY quantitation results mirrored HP values in terms of whether samples produced low or high DNA yield, though the exact values differed.

4.3. Real Time Quantities

Real Time quantities required no manipulation after being analyzed by the 7500 software, results are as produced by the internal analysis, and listed in Table 3 alongside Q-TAT HP results.

A Pearson correlational analysis measures the linearity between two variables. In this study, the analysis was applied to Q-TAT and Real Time results to determine if there is a statistically significant difference between the two quantitative methods. This analysis yielded a coefficient of 0.49, as seen in Table 4:

Table 4: Correlational Analysis of Q-TAT and Real Time Data

The CORR Procedure

2 Variables: RTDNA QTATDNA

Pearson Correlation Coefficients, N=48
Prob > |r| under H0: Rho=0

	RTDNA	QTATDNA
RTDNA	1.00000	0.49166
RTDNA		0.00040
QTATDNA	0.49166	1.00000
QTATDNA	0.00040	

Variables in the analysis refer to the quantitative method used, either Q-TAT or Real Time PCR. Because the same extract was quantitated using each method, here N does not indicate the number of *total* extracts, but rather the number of quantitations performed.

Here the null hypothesis, H_0, is the hypothesis that there is no difference between quantitative methods Q-TAT and Real Time, whereas the alternative hypothesis, H_1, represents the hypothesis that there is a difference between the methods. The null hypothesis is rejected ($p=0.0004$, $\alpha=0.05$),. Thus, there is a significant different in the quantitative estimates produced by the two methods.

4.3.1. Variance Among Tissue Types

Looking now at variation among tissue types, only the four main types—breast, colon, liver, and lung—were statistically analyzed.[h] First, a split-plot analysis of variance (ANOVA) was performed, which allows for examination of quantities by tissue type at two levels, here the quantitative methods. The split plot design examines variability at two different levels, DNA yield within tissue type, and then among each quantitative method. Tissue type was set up within the split plot design as the main unit factor (MUF), and quantitative method (Q-TAT or Real Time) as the split unit factor (SUF). Results showed that the variation within tissue types was high. This is an expected conclusion when results are generated as a quantity or amount. To assuage this variation, the original quantitative values were statistically transformed using a square root transformation. The interaction between the MUF and SUF was insignificant, as were the main effects, as seen in Table 5:

[h] Due to the fact that only one sample was collected of each minor tissue type (brain and kidney) no statistical analysis was performed on them. One sample was not considered representative enough of a larger population to draw significant conclusions. Moreover, maintaining relatively equal sample numbers among tissue groups (main types consisted of either four or five samples) was important in producing a reliable statistical analysis.

Table 5: Test of Fixed Effects

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Type	5	12.6	1.02	0.4469
METHOD	1	70.6	0.3	0.5832
METHOD*Type	5	70.6	0.18	0.9706

This test demonstrates that there is no relationship between tissue type and DNA yield regardless of quantitative method used, however the absolute values between the two methods still differed greatly.

The test of effect slices (Table 6) shows the method by each tissue type, again displaying a lack of significance.

Table 6: Tests of Effects Slices

Tests of Effect Slices

Effect	METHOD	Type	Num DF	Den DF	F Value	Pr > F
METHOD*Type		BRAIN	1	70.6	0.03	0.8719
METHOD*Type		BREAST	1	70.6	1.09	0.3001
METHOD*Type		COLON	1	70.6	0.20	0.6544
METHOD*Type		KIDNEY	1	70.6	0.09	0.7662
METHOD*Type		LIVER	1	70.6	0.38	0.5370
METHOD*Type		LUNG	1	70.6	1.26	0.2647
METHOD*Type	QTAT		5	15.8	0.75	0.6010
METHOD*Type	RT		5	15.8	1.12	0.3895

Showing a breakdown of the tests of fixed effects (Table 5), the tests of effects slices reiterates similarities among DNA yield by tissue type.

For each combination of method and type, the means (MNDNA) and standard errors (SEDNA) are shown in Table 7. While at first glance the means do seem very different, the statistical method used to analyze this data introduces large standard errors, rendering even sizeable differences insignificant.

Table 7: Mean and Standard Error

Type	METHOD	MNDNA	SEDNA
BRAIN	QTAT	9.2167	1.1833
BRAIN	RT	7.7800	4.2600
BREAST	QTAT	33.6667	16.4006
BREAST	RT	33.7286	7.4835
COLON	QTAT	40.8444	12.7862
COLON	RT	45.2156	11.0708
KIDNEY	QTAT	4.8000	0.2333
KIDNEY	RT	2.4220	0.8340
LIVER	QTAT	6.8652	2.4909
LIVER	RT	13.6131	5.8041
LUNG	QTAT	66.9250	27.3240
LUNG	RT	76.9640	21.0158

Large MNDNA differences do not equate to statistically significant differences after analysis because of the blocking, or arrangement of experimental units into subgroups, used in analysis. The subgroup blocks here are the tissue types.

4.3.2. Wax Removal Study

Three extractions were performed in pairs to examine the effect of removing wax on the final DNA yield. Previous studies indicated that a larger amount of wax may result in a reduced yield[38], but most wax-removal is simply confined to the deparaffinizing step[38]. Taking this concept one step farther, wax was physically removed from the tissues on glass slides prior to DNA extraction. Opaque tissues with a well-defined border and a solid (rather than spotted) consistency were selected to make wax removal as straightforward as possible.

Liver tissue 5178, and lung tissue 5266 were tested. Five slides from each tissue were extracted with all wax present, and 5 slides were extracted with as much

wax physically removed from the slide as possible. For the most part, wax separated nicely from the tissue border when scraped off the slide with a razor. The excess wax was discarded and tissue was extracted as usual.

Quantitation results from this wax removal study are as follows:

Table 8. Wax-Removal Study Quantities

Sample Type	ID	Total tissue Area of Five Slices	Total Residual Wax of Five Slices	Mean Total DNA Extracted (pg)
Liver	5178 Pos	0.0077	0.00886	33.32
Liver	5178 Neg	0.00774	0	56.4
Lung	5266 Pos	0.01588	0.02907	56.00
Lung	5266 Neg	0.01677	0	94

Results of study comparing extractions performed with and without wax, where "Pos" indicates a sample positive for excess wax, and "Neg" indicates a sample from which wax has been removed. DNA quantities reported are as quantitated by Real Time PCR.

While more data points are desirable in order to perform a statistical analysis of this effect, preliminary results seen here suggest that less residual wax results in a higher DNA yield.

4.3.3. Inhibition Study

In order to determine if increased DNA yield from wax-negative samples was due to reduction of PCR inhibition from paraffin wax, a study was performed using sample 5178Pos. Several dilutions of this sample were run with the Real Time 7500. If the calculated DNA yield decreased along with the sample's dilution, then inhibition was determined to be unlikely. Results in Table 9 do suggest that paraffin is unlikely as a factor in decreased yield between wax-positive and wax-negative

samples due to PCR inhibition. Of course, inhibitors may be present during PCR in the tissue itself coming from fixation, or in other cases, staining. Furthermore, paraffin wax may still be a source of inhibition in other ways, such as by negatively effecting DNA extraction from the cell.

Table 9. Inhibition Study Series Dilution

Sample	Dilution	Quantity (ng/uL)
5178Pos	none	0.606
5178Pos	dil 2	0.317
5178Pos	dil 4	0.135
5178Pos	dil 8	0.0652
5178Pos	dil 16	0.0679
5178Pos	dil 32	0.0272

The steady decrease in DNA quantities with increasing dilutions suggests that paraffin wax is not a source of PCR inhibition. Quantitations were done with qPCR.

Chapter V

Discussion and Conclusions

Comparing Q-TAT and Real Time qPCR is not a straightforward endeavor. While quantitation is the main objective of each method, they differ in the point at which they quantitate respective to amplification. Furthermore, there is not a universal "best" choice, as the methods have different strengths. Discussing and assessing the results obtained from each method may help future researchers determine which technique to apply in their own research, and offer a look at a practical comparison of end-point and qPCR, rather than one inside the confines of a traditional method validation study. Ultimately, evaluating a quantitative method for a forensic DNA laboratory will depend primarily on being able to produce good STR profiles on a daily basis.

5.1. Q-TAT

The strength of end-point PCR, and Q-TAT in particular, lies chiefly in the method's cost efficiency. Developed "in-house" with custom primers, Q-TAT can be performed for much less than qPCR. For laboratories without a Real Time PCR

thermocycler, which are sold in the $40,000 range, the cost of acquiring the equipment and necessary reagents may exceed a strict budget. Q-TAT is an effective alternative to quantify DNA yield and can be run for about $9.00 per reaction using common forensic laboratory equipment like a thermocycler and genetic analyzer (Maven Analytical, Peyton, Colorado).

Q-TAT is surprisingly flexible as evidenced in this study by the use of the HP locus over AMEL and SRY. The basic gender-typing function is unique to the method and can certainly be useful when seeking to identify contamination. In this study, the results obtained with the HP locus were superior to those at the X and Y loci, as indicated by the consistently higher R^2 value in the HP standard curve. The HP locus may have performed better due to its smaller size: it is roughly 100bp smaller than the AMEL X and Y loci.[i 33] Where degradation is a concern, as it is with aged or embedded samples, longer genomic fragments tend to break down more quickly than smaller fragments, and its smaller size would therefore offer HP some protection against degradation, though certainly not immunity. The pRL plasmid in Q-TAT is an effective indicator of inhibition and has been shown to be extremely sensitive.[36]

As with most scientific methods, some sacrifices must be made in the name of cost-efficiency, and end-point PCR is no exception to this rule. Taking post-amplification quantitative measurements has been shown to have an inherently higher

---

[i] AMEL-X is 210 bp in length and AMEL-Y 216 bp, while the HPRT gene is 99 bp.[33]

coefficient of variability than that claimed for qPCR which we found to be true as well (results not shown).[7] The reported coefficient of variation for Q-TAT is around 35%.[31] For many research endeavors—including STR profiling—this rate is acceptable. However, studies with a focus on more precise quantitation may call for a more exact technique.

5.2. Real Time

Speed and amplification efficiency are two highlights when considering Real Time PCR for research use. The 7500 measures the efficiency of an amplification reaction based on the threshold cycle (C_T) of known DNA samples. This cycle is the amplification cycle number at which the accumulation of RFU from an amplicon logarithmically increases with each successive cycle. Efficiency is determined by plotting C_T for each dilution in the standard curve against a log DNA concentration, giving a slope of -3.32 at 100% efficiency.[40] As long as the slope is close to -3.3, efficiency is considered optimal[j] (see Figure 8). This slope can be improved by using a broader range of standards, and acts as an internal control for PCR inhibitors as they will reduce efficiency.

---

[j] Amplification efficiency is a vital indicator in quantitation procedures, as consistent efficiency in all samples is necessary for reliable comparison. Invariable amplification becomes even more important when comparing known to unknown samples, as seen in this research and in most crime lab quantitative procedures. Differences in reference and target DNA can result in the under- or over-estimation of the actual DNA quantities. [40]

The utility of Real Time qPCR can be increased by using primers that allow for detection of DNA degradation. Pairing a primer for a longer target sequence (150-200 bp) with one for a shorter amplification target (50-80 bp), as in Q-TAT, can suggest degradation in cases where the shorter sequence is amplified more than the longer sequence. This approach is based on the theory that longer DNA fragments degrade faster than shorter fragments.[42]

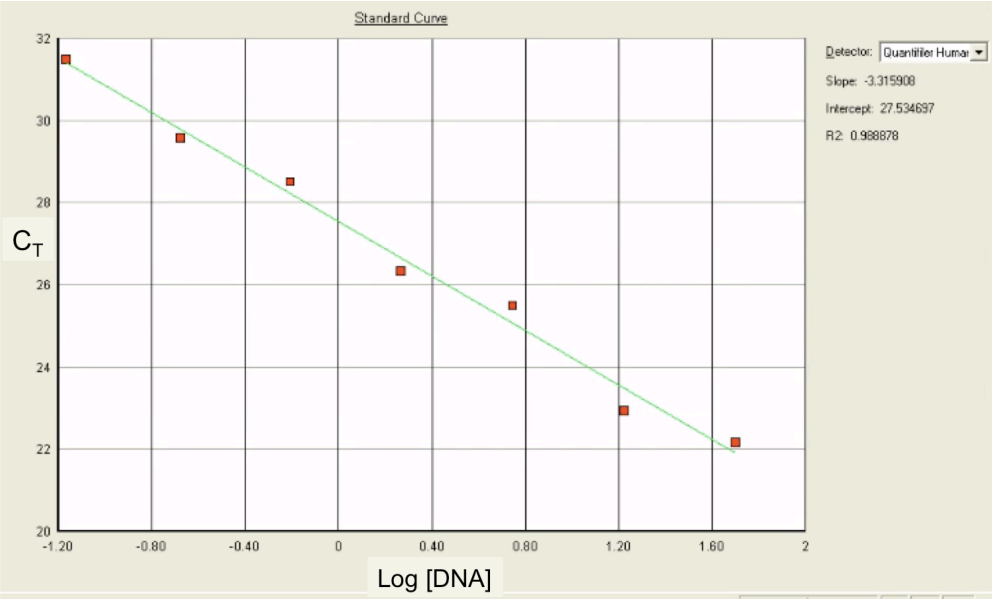


Figure 8: Efficiency Plot
Efficiency of a Real Time PCR reaction with C_T values plotted on the Y-axis against the log of the DNA concentration on the X-axis. The slope here of -3.3 indicates optimal reaction efficiency. The curve is fit by the Sequence Detection Software (SDS) version 1.2.3.[40]

The closed-tube system of qPCR also benefits an amplification reaction, as post PCR processing can allow for pipetting variability or introduce contamination into the quantitation procedure.[43]

Real Time is not without its disadvantages, however. Equipment such as the ABI Real Time 7500 can be cost-prohibitive, especially when considering validation and maintenance, and the purchase of proprietary reagents.[44] Some labs may still consider the initial equipment purchase an investment that can be recuperated in the reproducibility of results and time saved over end-point PCR.

5.3. Method Comparison

A correlational analysis of the quantitation estimates for DNA extracts from biological specimens produced using Q-TAT as opposed to Real Time showed the estimates to be significantly different, indicating the two methods yield estimates that differ. Statistical analysis only offers insight into the degree to which these two methods differ, rather than suggesting a superior quality of one method versus the other. Examining differences in these methods can hopefully help lead to a determination as to which method is called for in a particular situation. First, looking at the R^2 value can suggest a level of reliability for the method. This value measures the extent to which the standard DNA quantities adhere to a line of best fit, where an optimal value is 1.00. Real Time protocol calls for an R^2 of no less than 0.98, and all runs were at or above 0.98. Q-TAT R^2 values for the HP locus were in the range of 0.94 to 0.99. This is considered an acceptable range, but does leave room for improvement. The difficulty in achieving a higher R^2 value in Q-TAT necessitates a wider confidence interval for results.

There are a few reasons why the R^2 value for the end-point method may be lower, such as the error rate mentioned previously in section 5.1 *Q-TAT*. Post-amplification processing in end-point requires that samples be manipulated after PCR, when a sizing

standard is added to each sample. This involves pipetting 1µl of the amplicon into a clean tube, and mixing it with 24µl of the Liz/Hi-Di solution. Any additional processing step such as this may introduce enough human or mechanical error to account for a lower R^2.

A second factor of interest in comparing these two techniques is the point at which quantitation is determined. As an end-point PCR method, Q-TAT establishes a quantity for unknown values after the PCR procedure has completed and the amplicons are separated and quantitated on a genetic analyzer. Due to the nature of PCR amplification, the quantity of amplicons in identical samples may differ more at the end of cycling than at earlier phases. The early exponential phase of PCR is highly efficient, as each cycle exactly doubles the previous. However, reaction products begin to be consumed during the middle linear phase. Replicates then enter the final plateau phase at different rates and may even begin to degrade, resulting in lowered efficiency (Figure 9).[33]

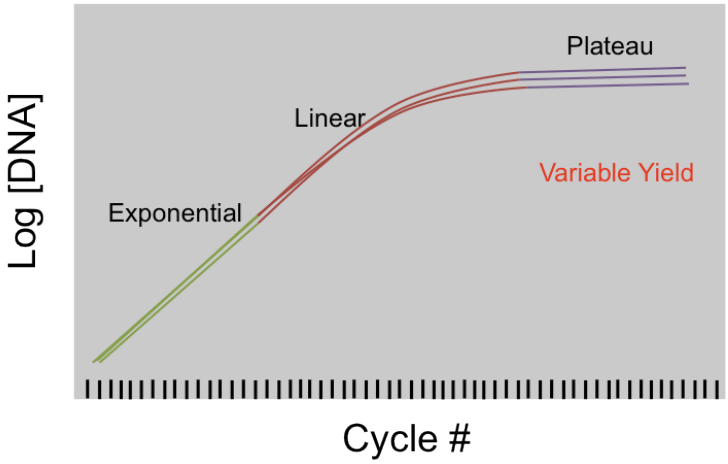


Figure 9: Quantitation of Replicate PCR Cycles
The quantity of replicate amplicons is very close during the exponential phase, but begins to spread as PCR progresses.[35]

Real Time PCR essentially resolves this issue by collecting data as soon as the reaction crosses the threshold, a point set in the exponential phase. The C_T is typically between 8 and 35 cycles into the PCR reaction. As a result of eliminating the post-PCR step, there is less chance for contamination and error to be introduced.

5.4. Tissue Type Comparison

While it was originally predicted that the density of certain tissues may correlate to the yield of recovered DNA, in practice the relationship of tissue type to picograms of DNA recovered was not statistically significant. Due to the great degree of variability in quantitative results, a statistical analysis of yield versus tissue type was difficult even after applying a square root transformation to the data. While five sections of tissue were used for each extraction, the amount of tissue in each section varied slightly owing to inconsistencies in the size of the original biopsy. Furthermore, variation in DNA yield

could be attributed to inconsistent cross-linking and fragmentation rates due to disparities in fixation times.

A large scale study may shed more light on DNA yield versus tissue type, as the fairly small sample pool studied here may not offer enough values to determine a reliable mean for each tissue group. Such a study could also be approached by selecting a uniformly sized extract from each section, rather than extracting five tissue sections and extrapolating an amount of DNA recovered per centimeter based on the measured area of the tissue. Alternatively, stained slides may offer an opportunity to calculate cell density prior to extraction and thereby craft a more specific measurement. Any effects staining may have on extraction should be taken into consideration.

This lack of significance is interesting on a number of levels, and it implies that tissue types do not need to be treated differently prior to extraction as previously suspected. This may be important when dealing with unknown tissue types or when processing a wide variety of tissues simultaneously.

5.5. Future Research

Despite some research into the area of DNA extraction from FFPE tissues, more work is needed. There are several avenues yet to explore, chief among them a broader tissue-differentiation study. A laboratory with more time to devote to the study may choose to investigate the idea of extraction tailored to tissue type in-depth, rather than the cursory level at which it was examined in this study.

Building upon the wax-removal study could offer more insight into possible benefits of this technique. Additionally, a study could explore more precise methods of removing excess wax, particularly from transparent or spotted tissue samples.

Enhancing the functionality of qPCR may be a goal for labs choosing to use this particular quantitation method, and research into primers that allow for degradation detection would benefit the scientific community as a whole.

Lastly, this study could be replicated using other FFPE extraction kits. The QIAmp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) is one viable option, and has been tested on some sections from the wax blocks that were used in this study. Early results as performed by Dr. Jun Fu (Human Identity Lab, OSU Center for Health Sciences) suggest that the QIAmp kit operates at a similar level as DEXPAT for incisional biopsies, but may offer improved DNA yield when using tissue samples from needle biopsies. A formal study is needed to generate conclusive results.

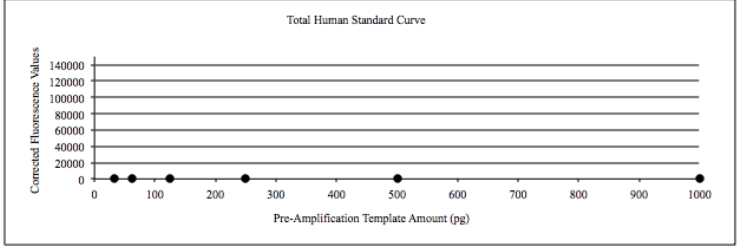
Appendix A

Area fluorescence values from the Applied Biosystems 310 GeneMapper software were entered into an excel spreadsheet, formulated to create a line of best fit through the data, the standard curve line:

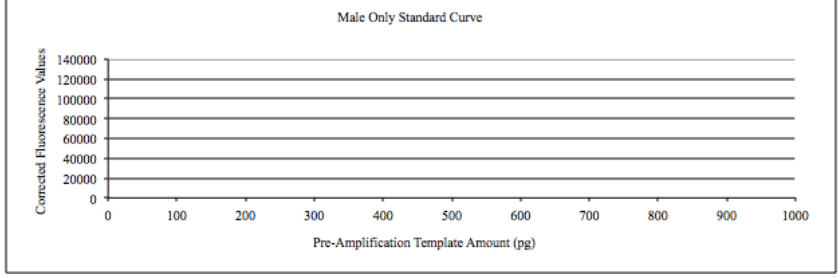
Sample DNA (pg)	X Fluorescence	Y Fluorescence	Average X + Y Fluorescence	SRY Fluorescence	Capillary Number	HP Fluorescnece
1000			0		C1	
500			0		D1	
250			0		E1	
125			0		F1	
63			0		G1	
32			0		H1	

Amelogenin X and Y values were summed to generate a Total Human DNA standard curve, while SRY and HP values were placed on separate graphs:

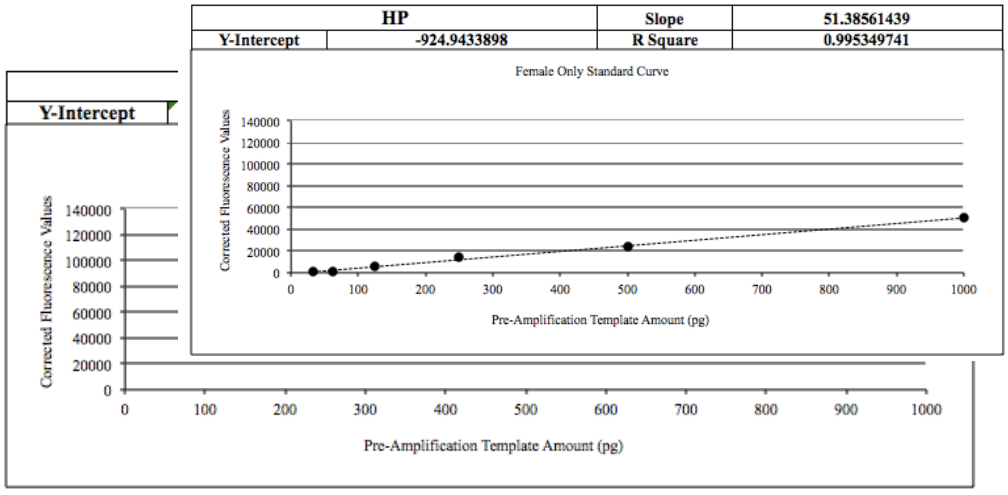
Total Human		Slope	0
Y-Intercept	0	R Square	#DIV/0!



Male Only		Slope	#DIV/0!
Y-Intercept	#DIV/0!	R Square	#DIV/0!



HP		Slope	51.38561439
Y-Intercept	-924.9433898	R Square	0.995349741



Using the calculated line equations, unknown values were entered into a separate chart, which generated DNA quantities in pg:

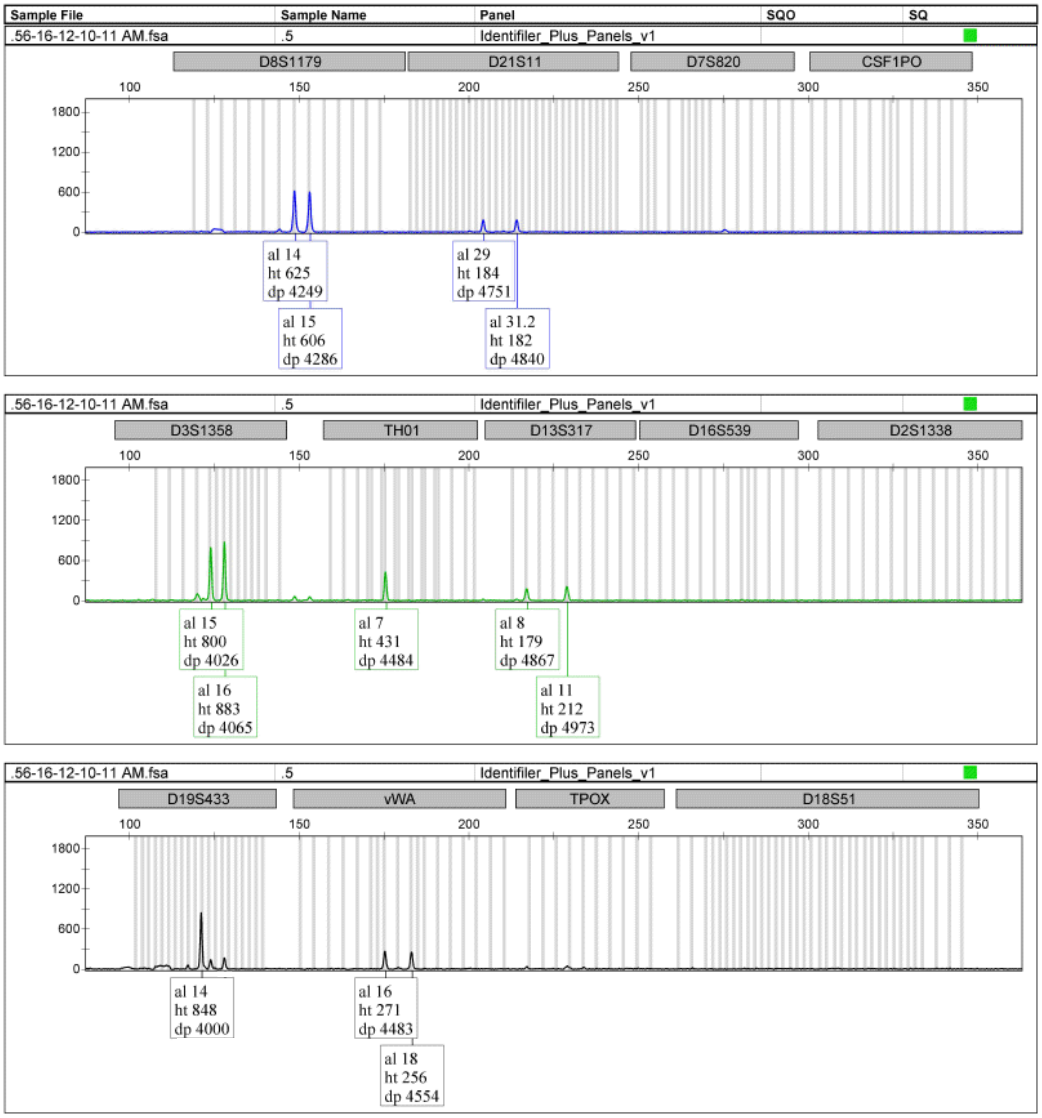
Sample	X	Y	X + Y	DNA (pg)
2445 (3.26)	783	0	783	**<32**
3968 (3.23)	3621	5696	9317	**72**
5178 (+)	0	1598	1598	**11**
5511 (3.26)	0	0	0	**<32**
6294 (3.23)	1039	636	1675	**12**
6829 (3.19)	766	1015	1781	**12**

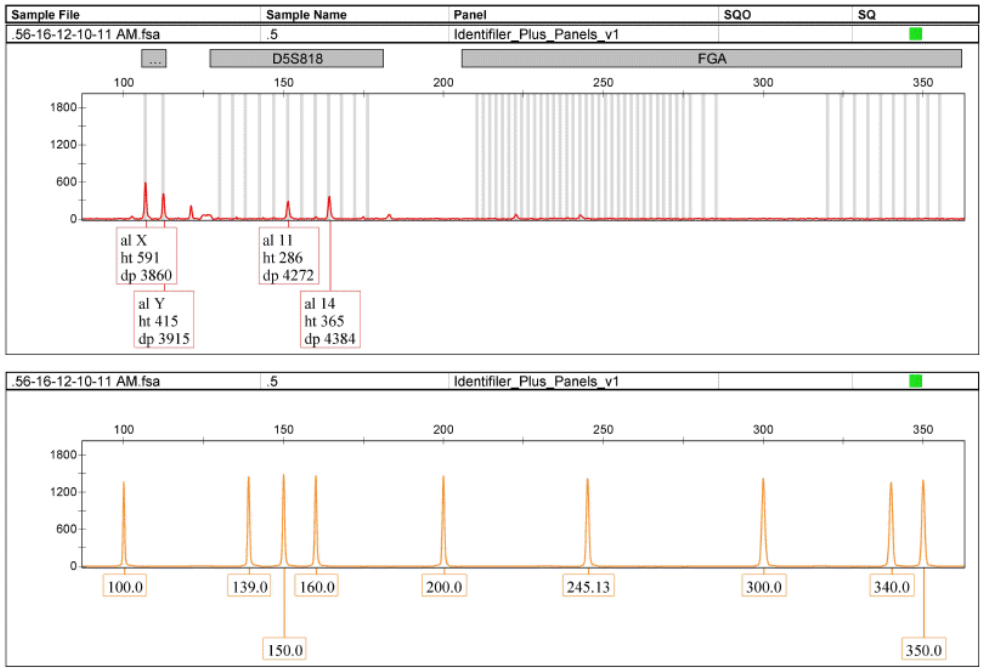
Sample	SRY	DNA (pg)
2445 (3.26)	0	**<32**
3968 (3.23)	39736	**528**
5178 (+)	12929	**149**
5511 (3.26)	0	**<32**
6294 (3.23)	14160	**166**
6829 (3.19)	9219	**96**

Sample	HP	DNA (pg)
2445 (3.26)	18820	**179**
3968 (3.23)	62867	**910**
5178 (+)	17874	**163**
5511 (3.26)	1885	**<32**
6294 (3.23)	37004	**481**
6829 (3.19)	16183	**135**

Appendix B

Successful DNA extraction from FFPE tissues was important in this study as a precursor to quality STR profiling. Therefore, a profile was developed to demonstrate the results that could be expected from biopsy sample, and also to validate quantitation results. The profile below was generated from five sections of lung biopsy sample 6829, which consistently yielded DNA quantities sufficient for STR typing. While this profile displays the downward RFU slope characteristic of degradation (as explained in section 2.5.5 *Degradation*), the alleles present are still useful for identification purposes.


Sample File
Sample Name
Panel
SQO
SQ
.56-16-12-10-11 AM.fsa
.5
Identifiler_Plus_Panels_v1
D8S1179
D21S11
D7S820
CSF1PO
100
150
200
250
300
350
1800
1200
600
0
al 14
ht 625
dp 4249
al 15
ht 606
dp 4286
al 29
ht 184
dp 4751
al 31.2
ht 182
dp 4840
.56-16-12-10-11 AM.fsa
.5
Identifiler_Plus_Panels_v1
D3S1358
TH01
D13S317
D16S539
D2S1338
100
150
200
250
300
350
1800
1200
600
0
al 15
ht 800
dp 4026
al 16
ht 883
dp 4065
al 7
ht 431
dp 4484
al 8
ht 179
dp 4867
al 11
ht 212
dp 4973
.56-16-12-10-11 AM.fsa
.5
Identifiler_Plus_Panels_v1
D19S433
vWA
TPOX
D18S51
100
150
200
250
300
350
1800
1200
600
0
al 14
ht 848
dp 4000
al 16
ht 271
dp 4483
al 18
ht 256
dp 4554


Sample File
Sample Name
Panel
SQO
SQ
.56-16-12-10-11 AM.fsa
.5
Identifiler_Plus_Panels_v1
...
D5S818
FGA
100
150
200
250
300
350
1800
1200
600
0
al X
ht 591
dp 3860
al Y
ht 415
dp 3915
al 11
ht 286
dp 4272
al 14
ht 365
dp 4384
.56-16-12-10-11 AM.fsa
.5
Identifiler_Plus_Panels_v1
100
150
200
250
300
350
1800
1200
600
0
100.0
139.0
150.0
160.0
200.0
245.13
300.0
340.0
350.0

References

1. Gephardt GN, Zarbo RJ. Extraneous Tissue in Surgical Pathology. *Archives of Pathology and Laboratory Medicine*. 1996;120:1010.

2. Worsham MJ, Wolman SR, Zarbo, Richard J. Molecular Approaches to Identification of Tissue Contamination in Surgical Pathology Sections. *The Journal of Molecular Diagnostics*. 3(1):11–15.

3. Sato Y, Mukai K, Matsuno Y, et al. The AMeX method: a multipurpose tissue-processing and paraffin-embedding method. II. Extraction of spooled DNA and its application to Southern blot hybridization analysis. *Am J Pathol*. 1990;136(2):267–271.

4. Hosoi H, Hirosawa S, Yamamoto K, Ohkusa T, Aoki N. Using Biopsy Materials to Detect Mutations in Gastrointestinal Tumors. *Journal of Clinical Gastroenterology*. 1995;20(4):272–6.

5. Crumlish M, Diab AM, George S, Ferguson HW. Detection of the bacterium Flavobacterium psychrophilum from a natural infection in rainbow trout, Oncorhynchus mykiss (Walbaum), using formalin-fixed, wax-embedded fish tissues. *Journal of Fish Diseases*. 2007;30:37–41.

6. Shi SR, Cote RJ, Wu L, et al. DNA Extraction from Archival Formalin-fixed, Paraffin-embedded Tissue Sections Based on the Antigen Retrieval Principle: Heating Under the Influence of pH. *Journal of Histochemistry & Cytochemistry*. 2002;50(8):1005–11.

7. Wickham CL, Boyce M, Joyner MV, et al. Amplification of PCR products in excess of 600 base pairs using DNA extracted from decalcified, paraffin wax embedded bone marrow trephine biopsies. *Journal of Clinical Pathology*. 2000;53:19–23.

8. Anon. Unlocking Your FFPE Archive: Critical factors for molecular analysis of FFPE samples. 2012.

9. Iwamoto S. DNA-based identification resolved suspected misdiagnosis due to contaminated cytological specimens. *Legal Medicine*. 5(4):246–50.

10. Rivero ER, Neves AC, Silva-Valenzuela MG, Sousa SO, Nunes FD. Simple salting-out method for DNA extraction from formalin-fixed, paraffin-embedded tissues. *Pathology, Research and Practice*. 2006;202(7):523–9.

11. Barnett R, Larson G. A Phenol–Chloroform Protocol for Extracting DNA from Ancient Samples. In: Shapiro B, Hofreiter M, Walker JM, eds. *Ancient DNA*.Vol 840. Methods in Molecular Biology. Humana Press; 2012:13–19. Available at: http://www.springerlink.com/content/hv56230569205m05/abstract/. Accessed May 12, 2012.

12. Qiagen. Multiple Displacement Amplification. *Sample & Assay Technologies*. Available at:

http://www.qiagen.com/products/bytechnology/wholegenomeamplification/tutorial/fixed.aspx.

13. Tokuda Y, Nakamura T, Satonaka K, et al. Fundamental study on the mechanism of DNA degradation in tissues fixed in formaldehyde. *J Clin Pathol*. 1990;43(9):748–751.

14. Miething F, Hering S, Hanschke B, Dressler J. Effect of Fixation to the Degradation of Nuclear and Mitochondrial DNA in Different Tissues. *Journal of Histochemistry & Cytochemistry*. 2006;53(3):371–4.

15. Anon. Picric Acid. *StainsFile*. 2005. Available at: http://stainsfile.info/StainsFile/dyes/10305.htm.

16. Dubeau L, Chandler L, Gralow J. Southern Blot Analysis of DNA Extracted from Formalin-fixed Pathology Specimens. *Cancer Research*. 1986;46:2964–9.

17. Protocols Online. Haematoxylin Eosin (H&E) staining. *Protocols Online*. 2010. Available at: http://protocolsonline.com/histology/haematoxylin-eosin-he-staining/.

18. Lounsbury JA, Coult N, Miranian DC, et al. An enzyme-based DNA preparation method for application to forensic biological samples and degraded stains. *Forensic Science International: Genetics*. (0). Available at: http://www.sciencedirect.com/science/article/pii/S1872497312000403. Accessed May 12, 2012.

19. Simons J, Vintiner SK. Effects of Histological Staining on the Analysis of Human DNA from Archived Slides. *Jounrnal of Forensic Science*. 2011;56(S1):S223–8.

20. Murase T, Inagaki H, Eimoto T. Influence of Histochemical and Immunohistochemical Stains on Polymerase Chain Reaction. *Modern Pathology*. 2000;13(2):147–151.

21. McCord B, Opel K, Funes M, Zoppis S, Jantz LM. An investigation of the effect of DNA degradation and inhibition on PCR amplification of single source and mixed forensic samples. 2011. Available at: https://www.ncjrs.gov/pdffiles1/nij/grants/236692.pdf. Accessed May 12, 2012.

22. Kosel S, Graeber MB. Use of neuropathological tissue for molecular genetic studies: parameters affecting DNA extraction and polymerase chain reaction. *Acta Neuropathology*. 88:19–25.

23. Zhang L, Wu Q. Single gene retrieval from thermally degraded DNA. *Jounral of Bioscience*. 2005;30(5):599–604.

24. Thompson WC, Ford S, Doom T, Raymer M, Krane DE. Evaluating forensic DNA evidence: Essential elements of a competent defense review. 2003. Available at: http://www.bioforensics.com/articles/champion1/champion1.html.

25. RGCB. RGCB - Regional Facility for DNA Fingerprinting. *RGCB*. 2012. Available at: http://rgcb.res.in/dnafingerprinting.html.

26. TaKaRa Bio. TaKaRa DEXPAT Easy; DNA Extraction from Paraffin-embedded Tissue Easy. Available at: http://www.takara.co.kr/file/manual/pdf/9104_e.v1010Da.pdf.

27. Werner M, Chott A, Fabiano A, Battifora H. Effect of Formalin Tissue Fixation and Processing on Immunohistochemistry. *American Journal of Surgical Pathology*. 2000;24(7):1016–9.

28. Alkali Scientific LLC. Zymo-Spin IIN. *AlkaliScientific*. 2012. Available at: http://www.alkaliscientific.com/en/zyppy-miniprep-kit-components/613-zymo-spin-iin.html.

29. Liu C, Wang M, Zhang T, Sun H. DNA hydrolysis promoted by di- and multi-nuclear metal complexes. *Coordination Chemistry Reviews*. 2004;248(1–2):147–168.

30. Sreedhara A, Cowan JA. Catalytic hydrolysis of DNA by metal ions and complexes. *J. Biol. Inorg. Chem.* 2001;6(4):337–347.

31. Allen RW, Fuller VM. Quantitation of Human Genomic DNA through Amplification of the Amelogenin Locus. *Journal of Forensic Science*. 2006;51:76–81.

32. Wilson JP. Developmental Validation of an Improved Multiplex Assay for Use in Forensic Casework: The Simultaneous Gender-Typing and Quantification of Total Human and Male-Only DNA within Forensic Samples. 2008.

33. Vandegrift E. Quantitation and Characterization of Human Nuclear and Mitochondrial DNA with PCR and Capillary Electrophoresis. 2010.

34. Smith BC. Evaluating DNA Sample Dagradation with a Quantitative Gender Typing End-Point PCR Multiplex. 2011.

35. Applied Biosystems. Real-Time PCR Vs. Traditional PCR. Available at: http://www6.appliedbiosystems.com/support/tutorials/pdf/rtpcr_vs_tradpcr.pdf. Accessed May 29, 2012.

36. Benson GA. *Improved Quantitation of Human DNA Using Quantitative Template Amplification Technology*. ProQuest; 2007.

37. D'Aquila RT, Bechtel LJ, Videler JA, Eron JJ, Gorczyca P, Kaplan JC. Maximizing sensitivity and specificity of PCR by pre-amplification heating. *Nucleic Acids Res*. 1991;19(13):3749.

38. An SF, Fleming KA. Removal of Inhibitor(s) of the Polymerase Chain Reaction from Formalin Fixed, Paraffin Wax Embedded Tissues. *J Clin Pathol*. 1991;44(11):924–927.

39. Chung J-Y, Braunschweig T, Hewitt SM. Optimization of Recovery of RNA From Formalin-fixed, Paraffin-embedded Tissue. *Diagnostic Molecular Pathology*. 2006;15(4):229–236.

40. Pfaffl MW. Determination of real-time PCR amplification efficiency. *Gene-Quantificaion*. 2004. Available at: http://efficiency.gene-quantification.info/. Accessed June 11, 2012.

41. Applied Biosystems. Amplification Efficiency of TaqMan Gene Expression Assays. 2006.

42. Swango KL, Hudlow WR, Timken MD, Buoncristiani MR. Developmental validation of a multiplex qPCR assay for assessing the quantity and quality of nuclear DNA in forensic samples. *Forensic Science International*. 2007;170(1):35–45.

43. Gene Target Solutions. Real-Time PCR FAQs. *Gene Target Solutions*. 2012. Available at: http://www.genetargetsolutions.com/pages/Real%252dTime-PCR-FAQS-.html. Accessed June 11, 2012.

44. Wong ML, Medrano JF. Real-Time PCR for mRNA Quantitation. 2005.

VITA

Laurie Ariola

Candidate for the Degree of

Master of Science

Thesis: Quantitation of DNA Extraction from Wax-Embedded Biopsy Tissues Using Two Different Methods

Major Field: Forensic Sciences

Education:

Completed the requirements for the Master of Science in Forensic Sciences at Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma, in July, 2012.

Completed the requirements for the Bachelor of Science in Biochemistry and Molecular Biology at Oklahoma State University, Stillwater, OK, in May, 2008.

Name: Laurie Ariola Date of Degree: July 2012

Institution: Oklahoma State University CHS Location: Tulsa, Oklahoma

Title of Study: QUANTITATION OF DNA FROM WAX-EMBEDDED BIOPSY TISSUES USING TWO DIFFERENT METHODS

Pages in Study: 65

Candidate for the Degree of Master of Science

Major Field: Forensic Sciences (DNA)

Scope and Method of Study: This study compares DNA yield from formalin fixed biopsy embedded tissues as quantitated by both Quantitative Template Assay Technology (Q-TAT) and Real Time qPCR. Additionally, the study examines the possibility of the effect tissue type may have on DNA yield. Major tissue types examined include breast, colon, liver, and lung biopsies, all embedded in paraffin wax after formalin fixation, and all roughly ten years old. Biopsy samples were sliced in 10μm thick sections and extracted in sets of five with TaKaRa DEXPAT reagent, followed by quantitation with both Q-TAT and Real Time.

Findings and Conclusions: The results show that the two quantitative methods produce significantly different results, and that tissue type does not have a statistically significant difference on DNA yield. Applications for both Q-TAT and Real Time are discussed, as they may each be preferred in different situations.

ADVISER'S APPROVAL: Robert Allen