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Identification of Human Blood Messenger Ribonucleic Acids through Non-Polymerase Chain Reaction Based Multiplexing

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Identification of Human Blood Messenger Ribonucleic Acids through Non-Polymerase Chain Reaction Based Multiplexing

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Abstract

The goal of this research project was to develop a method for the identification of human blood that was simple and fast to use, yet sensitive and specific enough for forensic casework. Based on these criteria, the ideal assay would be based on messenger ribonucleic acid (mRNA) multiplexing specific for blood identification. According to the central dogma of molecular biology and gene expression, it can be theorized that the identity of human specific biological material can be obtained based only on the mRNA expressed by genes of a particular tissue. Once isolated, the tissue specific mRNA can be detected using the affinity of deoxyribonucleic acid (DNA) probes which contain the complementary sequence for annealing. The newly annealed double stranded nucleic acid will then serve as the target for detection via fluorescent molecular labeling, which will allow the human specific biological material targets to be detectable under ultraviolet light. Development of such a method would provide the field with a more rapid and accurate assay for analysis of forensic serology samples.

Keywords: mRNA, blood identification, forensic serology

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Introduction

Biological evidence, most often blood, is frequently left at crime scenes and discovered by forensic scientists that subsequently attempt to obtain the genetic profile from the DNA contained within the stain. The genetic profiles obtained from these crime scene samples are then compared to the genetic profiles of known suspects and victims. Based on the genetic evidence obtained, the forensic scientist reports the results of the analysis. The field of forensic DNA analysis has reshaped the face of the criminal justice system like few forensic disciplines before it, and, as a result, the field of forensic serology has become marginalized. What is often forgotten is the role that biochemical and immunological tests play during the course of a criminal investigation. The modern field of forensic serology provides a method for the identification of some but not all forensically relevant body fluids. As important as body fluid identification is, some of the forensic serological tests are time consuming, labor intensive and too frequently report false positives. It was the goal of this research project to investigate the possible use of a non-polymerase chain reaction (PCR) based method for the identification of human blood.

Brief History of Forensic Biology

According to Kiel (1970), the first known article on the topic of forensic medicine was written by the Chinese doctor Zhicai Xu in the 6th century entitled *Ming Yuan Shi Lu*. In approximately 1250, Ci Song, an official of criminal justice administration in China, compiled a more comprehensive and consequently better-known dissertation on legal medicine entitled *Xi*

Yuan Lu or *Instructions to Coroners*. In addition, Kiel noted that the first known reference to the detection of blood was mentioned in *Xi Yuan Lu*. In *Instructions to Coroners*, Ci Song wrote that an old bloodstain on a knife was detected by heating the stain and treating it with vinegar. The resulting change in the bloodstain's appearance, from red to brown, supported that the stain was blood. A version of the method described by Song is still used today, and it is understood that the color change is due to the formation of hematin crystals in the sample. Modern forensic serologists recognize that the described method results in a reaction between the hemoglobin in the blood and the acetic acid in the vinegar. It is this simple "vinegar test" that is considered to be a rudimentary version of the Teichmann crystal test.

In the early to mid-nineteenth century, the field of forensic serology advanced substantially with the development of the first presumptive/screening test. In 1818, Louis Jacques Thénard discovered hydrogen peroxide (Thénard, 1818) and subsequent to his discovery, other scientists began to describe the peroxidase-like activity of blood hemoglobin, which most presumptive tests are relied on. By 1863, Christian Friedrich Schönbein recommended the use of the guaiacum test to serve as a presumptive test for blood (Schönbein, 1863). A positive result on the guaiacum test was observed as the formation of bubbles after hydrogen peroxide was applied to the blood stain. While the guaiacum test was prone to false reporting, it is a notable contribution to the history of forensic serology because it advanced the way investigators perceived biological evidence.

The most familiar and widely utilized presumptive test for the identification of blood may be the benzidine test, which was introduced by Oscar and Rudolf Adler (1904). Benzidine forms a color indicator intermediate product called "benzidine blue" as the chemical reaction between

peroxide and blood moves forward. The benzidine blue intermediate has a more desirable and recognizable color change than the end product “benzidine brown”, and has a maximum stability at pH 4.5. Due to the stability of the intermediate product in an acidic environment, the benzidine oxidation reaction by peroxide or the peroxidase-like activity in blood is carried out at an acidic pH. The benzidine test is very sensitive, and it has shown the ability to detect the presence of blood in a $1{:}10^5$ dilution with water (Adler & Adler, 1904). The use of benzidine has been abandoned in many laboratories due to the fact that it is a known chemical carcinogen. Over time, less toxic benzidine derivatives have been used in place of benzidine. In addition, other chemical tests for locating blood such as the phenolphthalein and luminol tests are more frequently used.

Around the turn of the 20th century, the focus of forensic serology changed from locating biological stains to identifying from whom the body fluid originated. The discovery of chemical interactions between antigens and antibodies by Paul Ehrlich and Johannes Morgenroth (1899b and 1901) made it possible to identify immunological blood groups, and ushered in a new era of serology and forensic serology testing. From this discovery the field of serology, as a branch of laboratory medicine that evaluates blood sera, flourished and grew. New serological techniques made it possible to measure an individual’s serum antibody titers against a variety of different antigens associated with diseases. The medical applications of serology were numerous, but it was the discovery of ABO blood grouping that benefited forensic science most.

The most important breakthrough for forensic serological blood grouping was the discovery of ABO blood groups by Karl Landsteiner (1901). The discovery of the ABO blood groups not only made blood transfusions and organ transplants possible, but also allowed forensic

scientists to determine the ABO blood group of blood found at a crime scene, which aided in the elimination of individuals from the suspect pool. One limitation to Landsteiner's research was that his method of classification depended on intact red blood cells and their corresponding antiserum. If the red blood cells within a blood sample were destroyed, then there was no way to identify the stain. Fortunately, Leone Lattes resolved this problem in his book published in 1923 titled *L'Individualità del Sangue Nella Biologia, Nella Clinica, Nella Medicina, Legale,* or *Individuality of the Blood in Biology and in Clinical and Forensic Medicine*. In this book, Lattes focused on dealing with clinical issues, heritability, and the typing of dried stains. Lattes also introduced the idea that antibodies are still active even though the cells in a sample are not intact. His revelation ultimately resulted in the determination of blood grouping by identifying the presence of antibodies instead of complete cells. Although Lattes' test still has significant drawbacks, such as small quantities or degenerated stains are shown to result in false positives and the test lacks sensitivity, Lattes' theory was a dramatic improvement for forensic serology.

In 1925, Saburo Sirai discovered that group-specific antigens were secreted into body fluids other than blood (Sirai, 1925). For forensic investigators, this meant that a seminal fluid or saliva sample found at a crime scene could be as probative to a case as blood. Subsequent to the work of Sirai, Yamakami (1926) validated that group-specific substances could be found in semenal fluid, saliva and vaginal secretions. In that same year, Landsteiner and Levine (1926a) found that blood group specific elements were present in spermatozoa and paralleled the result of blood analysis. Over the next few years blood group specific antigens were also found in tears, pericardial fluids, concentrated urine and organ cells (Yosida 1928a and 1928b; Thomsen 1930a

and 1930b). With the expansion of studies on group-specific antigens in other body fluids, the detection of additional blood factors became more sensitive. Landsteiner and Levine (1927a and 1927b) detected the M, N, and P blood factors that composed MNSs and P typing systems. In 1940, Landsteiner and Wiener presented their research about Rh blood group (Landsteiner & Wiener, 1940). In 1946, Mourant discovered the Lewis blood group system (Mourant, 1946) and the Kell blood group system was introduced by R.R. Race (Race, 1946). Taken in combination, these findings contributed significantly in the production of a powerful series of blood group assays that could be used to aid an investigation. However, these traditional forensic serology techniques cannot uniquely individualize persons. It was not until the development of forensic DNA analysis in the 1980s that forensic investigators were able to examine and identify the origin of a stain using the DNA present in the biological fluid.

The discovery of DNA and the emergence of forensic DNA analysis dramatically reshaped the face of forensic serology. Up until the late 1980's, forensic serology employed immunological tests to differentiate between people and narrow down the number of potential suspects. After the implementation of forensic DNA analysis, forensic serology was transformed into the applied science of locating and characterizing forensically relevant body fluids for downstream DNA analysis. Although forensic serology shares a common theory with classical serology, modern forensic serology serves the function of locating forensically relevant body fluids, such as blood, semen, saliva, urine, feces, vaginal secretions, and menstrual blood efficiently and at minimal cost.

Gregor Mendel's research on genetic inheritance went largely unnoticed even after he published his findings in 1865 (Mendel, 1865). It was not until Bateson (1909) "rediscovered"

Mendel's work that the idea of an inheritable molecule took root in the scientific community. By 1984, the first DNA profiling test by multi-locus RFLP (restriction fragment length polymorphism) was introduced to the world. Dr. Alec Jeffreys and his colleagues published their work in *Nature* in 1985 (Jeffreys, 1985), which resulted in a steady stream of requests for DNA analysis by individuals and law enforcement agencies. The first application of DNA technology in a criminal case was in 1986, where DNA evidence eliminated the primary suspect and lead to a screening involving several thousand male residents in central England. (Craig, 1988). By November 1987, DNA profiling was accepted in U.S. criminal courts, and the era of immunological forensic serology ended. (Andrews v. State, 1988)

Decades after the first forensic DNA case, the familiarity of DNA has permeated society. The current aim of forensic serology is to analyze a sample from a crime scene and identify the type of body fluid it is, so it may be associated with the results of the DNA test. As RNA is the intermediate between DNA and protein in the forensically relevant body fluids, it holds the key to differentiate fluids and tissues, current advances in technology have provided the forensic community with the opportunity to evaluate the RNA within a body fluid, and preliminary data indicates that such a methodology could result in a molecular means to identify forensically relevant biological fluids.

Forensic Serology - Presumptive Tests for the Discovery of Blood

In forensic serology, tests for the identification of blood are categorized as presumptive tests, confirmatory tests, species identifying, and grouping. A presumptive test is an assay that

provides chemical indications of the possible existence of a specific biological material. Presumptive tests are most often catalytic and provide results within seconds, making them ideal for screening biological evidence efficiently. Over the years, a number of presumptive tests for various biological materials have been developed, and each one has its own strengths and weaknesses.

The phenolphthalein, tetra-methyl benzidine, o-tolidine, and leucomalachite green tests belong to the category of catalytic test which rely on the peroxidase-like catalytic property of hemoglobin. Hemoglobin is a tetrameric molecule composed of two alpha globin chains, two beta globin chains, and four iron-containing heme groups. Hemoglobin is a large protein contained within red blood cells that is responsible for binding and transporting oxygen throughout the circulatory system of most vertebrates. (Maton, 1993) The unique function of hemoglobin and the fact that it is found exclusively in red blood cells makes the heme in hemoglobin ideal for forensic applications as heme possesses the peroxidase-like activity. However, hemoglobin is not enzyme because enzyme is defined as protein have *in vivo* catalytic function. Hemoglobin is able to catalyze the peroxide-mediated oxidation of the presumptive test reagent as hemoglobin would oxidize hydrogen peroxide into water and oxygen, the oxygen produced would then lead the oxidation reaction (Mahler & Cordes, 1971). In forensic applications, the peroxidase-like activity of hemoglobin catalyzes the oxidation of a number of organic compounds which yield a change in coloration from the starting reagents to the resulting product (Gaensslen, 1983).

The phenolphthalein test is one of the most popular presumptive tests used in crime laboratories. Kastle and Shedd (1901) first introduced the phenolphthalein test and showed that

“oxidizing ferments” within the blood would catalyze the oxidation of colourless phenolphthalin to pink phenolphthalein. Five years later Kastle and Amoss (1906) elucidated that the color change reaction relied on the peroxidase-like activity of hemoglobin. In the presence of phenolphthalin, the activity of hemoglobin causes the division of hydrogen peroxide into hydrogen and water. As such, the oxidation of phenolphthalin results in the production of phenolphthalein. Kastle and Amoss also determined that the observed catalytic activity is proportional to the concentration of hemoglobin sampled (Kastle & Amoss, 1906).

Like most serological presumptive tests, the phenolphthalein test is very sensitive, and has been shown to detect blood in a 10^6 dilution. (Deléarde & Benoit, 1908; Girdwood, 1926) However, the high degree of sensitivity contributes to the frequency of false positive results by forensic presumptive tests. The phenolphthalein test, like many other presumptive tests, gives false positives to many biological and chemical materials (Pozzi-Escot, 1908). For example, methemoglobin, hematin chlorhydrate, saliva, pus, malt extract, vegetable extracts and heavy metal salts all generate false positives (Deléarde & Benoit, 1908). The sensitivity of forensic presumptive tests must therefore be coupled with the specificity of a confirmatory test.

The examination of biological evidence using presumptive tests is vital to the success of any forensic investigation. The functionality of presumptive tests is to aid in the timely screening of biological evidence. Therefore, a quality presumptive test must be inexpensive, simple to perform, and cannot interfere with subsequent tests. In addition, a result should be timely, and the resulting color change should be obvious to the analyst. As useful as presumptive tests are, they lack specificity and require additional confirmatory testing before a conclusive result can be made.

In short, current presumptive tests do not offer both a high degree of sensitivity and specificity, and additional research into the field of forensic serology is needed.

Forensic Serology - Confirmatory Tests for the Identification of Blood

In the field of forensic serology, confirmatory tests are used to identify a given biological material to the exclusion of all other possibilities. There are several confirmatory tests currently used by forensic analysts. These tests are limited to the analysis of blood and semen, such as the hemochromogen crystal test, ABAcard® HemaTrace test for blood identification, and the microscopic identification of spermatozoa.

Masaeo Takayama (1912) demonstrated that hemoglobin in the blood, when exposed to pyridine, would react and produce feathery, pink hemochromogen crystals. In his orginal publication, Takayama proposed the use of saturated dextrose, 10% NaOH, pyridine and water to achieve the formation of hemochromogen crystals. While it has been conclusively determined that the sugar in Takayama reagents is not essential, both Dilling (1926) and Kerr (1926a) concluded that the use of sugar decreased the solubility of newly formed hemochromogen crystals making them easier to visualize. In addition, the application of gentle heat often resulted in the best results. The speed of crystal formation was determined to be dependent on the temperature and freshness of the Takayama reagents. The higher the temperature the faster the appearance of hemochromogen crystals.

Although the hemochromogen crystal test is blood (hemoglobin) specific and moderately sensitive, a negative result should not be inferred as the absence of blood (Kirk, 1953; Olbrycht,

1950). When a bloodstain becomes exposed to heat, weather, or too old, the hemoglobin can become difficult to solubilize, and crystal formation becomes challenging (Katayama, 1888). Moreover, the hemochromogen crystal test can only confirm the presence of blood hemoglobin, and lacks the ability to determine the species or individual of origin (Takayama, 1912). The possibility of a false positive result for the hemochromogen crystal test was investigated by Blake and Dillon (1973). Concerns about whether other iron-protoporphyrin containing substances, like catalase and peroxidase, would falsely produce positive results were of specific interest. Results obtained from the work of Blake and Dillon (1973) demonstrated that both purified catalase and peroxidase produced false positive results when evaluated and that the crystals formed with the enzyme catalase were hard to distinguish from those obtained from whole blood. However, when naturally occurring catalase and peroxidase were evaluated no reaction was observed.

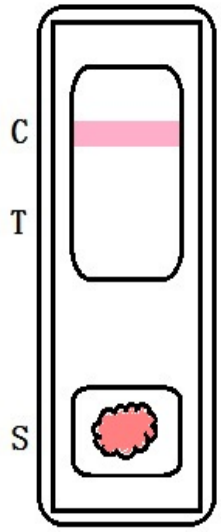

Figure 1. ABAcard® HemaTrace test strip. This figure illustrates a negative result showed on an ABAcard® HemaTrace test strip, “S” for sample loading area, “C” for control area and “T” for test area.

Recent advances in the field of forensic serology have resulted in the development of an immunologic based method for the identification of human hemoglobin. The ABAcard® HemaTrace test strip (Abacus Diagnostics, West Hils, CA) utilizes solubilized whole blood for the specific and prompt analysis of human blood. As showed in Figure 1, a solubilized blood sample can be loaded on to the sample well of the test strip. As the liquid sample migrates along the test strip, the human hemoglobin present in the sample forms a stable interaction with a dye-labeled

monoclonal antihuman hemoglobin antibody to form a mobile antibody-antigen complex which migrates through the absorbent membrane in the strip toward the test area. At the testing site, there is a localized population of stationary polyclonal antihuman antibodies. When the mobile antibody-antigen complex passes by the polyclonal antibodies at the test site, the mobile antibody-antigen complex forms an additional bond with the stationary antibodies resulting in an antibody-antigen-antibody complex. If the reaction is positive for blood, then a pink band will form at the test site labeled "T". This pink band is the result of the accumulation of dye labeled antibodies at the test site. Although the ABAcard® test is sensitive, specific, and rapid (Swander & Stiles, 1998), it has also been shown to react with primate and ferret hemoglobin. A false negative or weak result may also occur when the concentration of human hemoglobin in the sample is above the optimal level resulting in a phenomenon called high dose hook effect (Amarasiri Fernando, 1992).

The average confirmatory tests involve substantial investment of time and labor in order to perform the required analysis. In addition, confirmatory tests are only useful when a body fluid of interest possesses a unique chemical or physical property. Therefore, for the majority of forensically relative body fluids there is no confirmatory test available. While the development of new forensic serology techniques appear to be stagnating, the field of forensic DNA analysis has advanced to the point that it is now possible to determine most the likely contributors of the majority of biological stains recovered at a crime scene.

Molecular Nature of Forensic Identification

For more than thirty years, forensic DNA analysis has been utilized for human

identification. However, until recently little attention has been paid to the use of genetic information for the identification of body fluids. With the recent advances in molecular biology technology and armed with a better understanding of RNA, it appears that the field of forensic body fluid identification is poised to make the transition from a discipline based on immunologic interactions to one founded in the field of molecular genetics.

Chromosomes within all organisims contained both proteins and DNA. DNA was first discovered by Friedrich Miescher in 1869, and was named "nuclein" due to its location in the cell nucleus (Miescher, 1869a). By the end of the nineteenth century, both DNA and RNA were isolated and purified from a variety of different cells. Despite the amassing information about DNA, there was no way to show conclusively that DNA or any other molecule carried inheritable information. In fact, the primary opinion in the 1800s was that DNA merely served as a frame for the formation of the chromosome and that protiens were the molecules of inheritance.

Early in the twentieth century, Phoebus Levene identified adenine, guanine, thymine, and cytosine as the four-nucleotide monomers of DNA (Levene, 1919 and 1929). By 1928, Frederick Griffith reported that heat-killed fatal colonies of *Streptococcus pneumoniae* could transform avirulent bacteria to a virulent form, thus providing the first evidence that inheritable characteristics could be passed to a descendant by an internal cellular component (Griffith, 1928). In 1944, Avery, MacLeod and McCarty announced that DNA was the genetic molecule, based on evidence similar to the *S. pneumoniae* transformation experiment Griffith introduced. Avery *et al.* reported that the transformation of virulence was still achievable after all proteins were purified from the extraction (Avery, MacLeod & McCarty, 1944). In addition, DNA specific digestive

enzymes destroyed the activity to transform the target cell (McCarty, 1985). Altogether, the findings indicated that the transforming material was DNA. When it was finally made clear that DNA was the molecule of inheritance, studies by multiple research groups began to focus on understanding the structure and function of DNA and RNA.

Structure of DNA and RNA

Determining the structure of DNA and RNA took decades and the combined effort of a number of research groups. DNA and RNA belong to a class of macromolecules called nucleic acids, which are polymers comprised of numerous nucleotides. Nucleotides are the monomers of DNA and RNA and consist of a single phosphate molecule joined to a 5-carbon sugar ribose, to

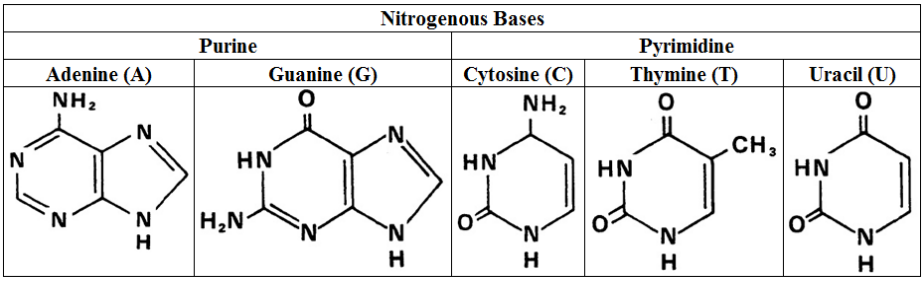

Figure 2. Nitrogenous bases of nucleic acids. This figure illustrates the structures of five nitrogenous bases that occur in nucleic acids, and their categories.

which one of five nitrogenous bases is attached. The five nitrogenous bases are further divided into two classes based on structure. The purines are adenine and guanine, which have a double-ringed structure; the pyrimidines are thymine, cytosine and uracil, with variations on the single-ring structure. The names of these five bases are commonly abbreviated to the first letter. Figure 2 highlights the similarities and differences between the five nucleotides. From figure 2, it can be observed that minor variations in chemical structure are responsible for the large amount of

physical diversity we see.

The five-carbon sugar that makes up the backbone of DNA is identified as a 2'-deoxyribose because it lacks a hydroxyl group at the 2' carbon. Unlike DNA, the 2' carbon of RNA is hydroxylated, and it is the presence of the 2' hydroxyl functional group that allows one to distinguish between DNA and RNA. The nitrogenous bases of both RNA and DNA form a glycosidic bond at the 1' carbon of the sugar and either the N1 of pyrimidines or N9 of purines. Additionally, phosphoric acid is esterified to the 5' carbon sugar, resulting in the formation of a functional nucleotide. The importance of the chemical structure is best observed in the stability of nucleotides. Once formed, the nucleotides within a cell are frequently recycled by the cell but rarely resynthesized.

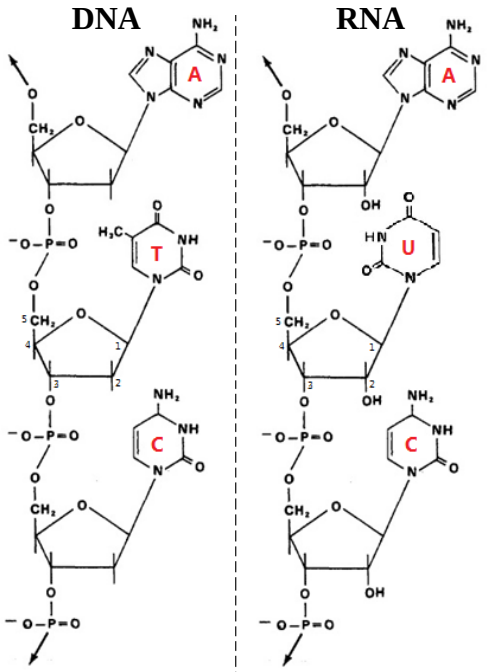

Figure 3. Polynucleotide chains. This figure illustrates the structures of a deoxyribose polynucleotide chain (left) and a ribose polynucleotide chain (right), with the atom position number.

Nucleotides are linked to each other to form long polynucleotide chains of either DNA or RNA. In figure 3, the deoxyribose (left) and ribose (right) polynucleotide chains are easily identifiable based on the presence of a hydroxyl functional group at the 2' carbon. It should be noted that the linkage between the 3'-hydroxyl group of the sugar from the first nucleotide and the 5'-phosphate of the next nucleotide results in the formation of a phosphodiester bond. Most importantly, the phosphodiester linkage between nucleotides establishes the repeating sugar-phosphate motif of the DNA or RNA backbone. While the

backbone of nucleic acids remains consistent, the nitrogenous bases, internal to the backbone, are highly variable, and are the fundamental method by which nucleic acids communicate the genetic information stored within genes. The average RNA molecule consists of a single nucleotide chain, similar to that illustrated in Figure 3, while DNA is the result of two-polynucleotide strands duplexed together.

DNA consists of A, C, T and G nucleotides, while RNA consists of A, C, U and G nucleotides. Erwin Chargaff (1951) reported that DNA from mammalian sources has an approximate 1:1 ratio of pyrimidine to purine. More specifically, the amount of A is equal to T, and the amount of G is equal to C, yet, the ratio of A and T to C and G varied between species. This finding became known as Chargaff's rule, and provided the first indications that A pairs to T and C pairs to G. In 1953, James Watson and Francis Crick published their double-helix model of DNA structure based on chemical and physical data from multiple sources (Watson & Crick, 1953). According to the Watson - Crick Model, two DNA strands entwine around each other to form an anti-parallel complementary helix. The regular repeating sugar-phosphate of the DNA molecule serves as the backbone, and the nitrogenous bases internal to each strand of the helix are configured in a complementary relationship. The complementary relationship provides the DNA molecule with the ability to self-encode, which allows the two strands to replicate and transcribe faithfully.

The complementary nature of nucleic acid is regulated by the formation of hydrogen bonds, as seen in figure 4. The interaction between A-T and A-U pairs results in the formation of two hydrogen bonds. The first hydrogen bond forms between the A-T and A-U nucleotides at the exocyclic amino group at C6 of adenine and the carbonyl group at C4 of thymine/uracil. The other hydrogen bond forms between N1 of adenine and N3 of thymine/uracil. The guanine and cytosine pairing results in the formation of three hydrogen bonds. The hydrogen bonds form at the exocyclic amino group of C4 on cytosine and the carbonyl at C6 of guanine, the N1 of guanine and N3 of cytosine, and finally between the exocyclic NH_2 at C2 of guanine and the carbonyl at C2 in cytosine. The ability to form complementary interactions via hydrogen bonding is a phenomenon that can be observed both *in vivo* and *in vitro,* and can occur in the formation of DNA-DNA, DNA-RNA, or RNA-RNA complexes.

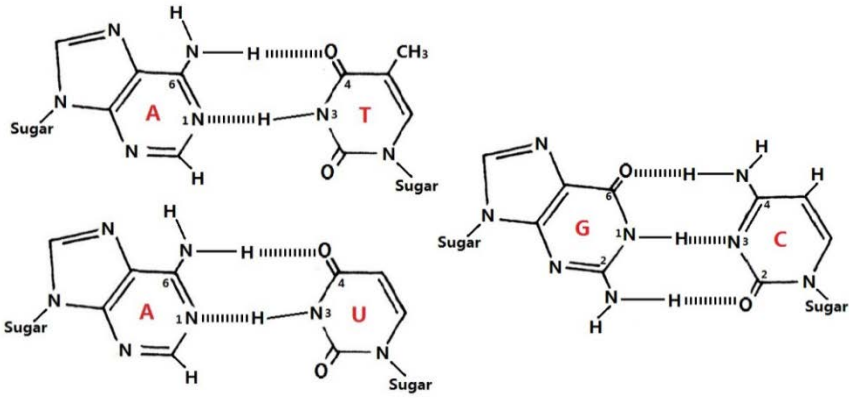

Figure 4. Base pairs. This figure illustrates the A-T, A-U and G-C base pairs and the hydrogen bonds between those pairs, with the atom position number.

DNA Denaturation, Reassociation and Hybridization

In 1958, Meselson and Stahl carried out a series of experiments that provided support to

the hypothesis that the two strands of the DNA molecule disassociate from each other during DNA replication (Meselson & Stahl, 1958). In addition, the complementary strands of the DNA helix were determined to be denaturable *in vitro* by applying substantial heat or high pH. When heat-denatured solutions of DNA were allowed to cool down slowly, the complementary strands reannealed and the double helix structure of DNA reformed. In a laboratory setting, DNA has been shown to reanneal and form artificial hybridizations between DNA and RNA of complementary sequence. The first documented example of DNA-RNA hybridization was described by Spiegelman in 1961 (Hall & Spiegelman, 1961). Since then, the use of hybridization technology has become a mainstay of molecular biology resulting in the creation of techniques such as polymerase chain reaction (Mullis, 1986), Southern blotting (Southern, 1975), and DNA microarray analysis (Augenlicht, 1982). During the course of this project the phenomena of DNA-RNA hybridization was evaluated for the possibility of detecting RNA markers specific for forensically relevant body fluids.

Transcription and Translation

In 1958, Francis Crick proposed the concept of “The Central Dogma of Molecular Biology” as an illustration of the fundamental relationship between DNA, RNA, and protein (Crick, 1970). According to Crick’s illustration, the genetic information contained within genes becomes converted into RNA and finally into protein. The synthesis of RNA from complementary DNA is known as transcription, and is an enzyme-catalyzed reaction under the direct regulation of RNA polymerase. RNA polymerase utilizes the principles of “Watson-Crick” paring to manufacture a

mobile copy of genes that is faithfully transcribed into a form of RNA.

Transcription has three phases: initiation, elongation, and termination. Initiation starts with the binding of RNA polymerase to the promoter region of a gene. The binding of the RNA polymerase molecule causes a physical change in the structure at the promoter-polymerase complex site, ultimately leading to the localized unraveling of the DNA helix at that location. Promoters serve as the initiation signal for transcription and, as the two DNA strands separate, the RNA polymerase catalyzes the first ten-phosphodiester bonds of a newly formed RNA chain before transitioning into the next phase. In the elongation phase, RNA polymerase moves along the DNA template exposing the bases of the DNA strand and incorporating the complementary ribonucleotide into the growing RNA strand. RNA polymerase is multifunctional during the process of elongation, not only directing the growth of the RNA chain, but also checking for transcription errors as it passes. Once the RNA polymerase meets the termination sequence encoded by the DNA, would results in termination of transcription. The RNA polymerase dissociates from the DNA template and results in the release of the RNA product.

Following transcription, RNA is exported into the cytosol of the cell where translation takes place. According to the central dogma of molecular biology, the genetic information encoded within messenger RNA (mRNA) is translated into protein. The successful translation of mRNA to protein requires additional cellular components in the form of transfer RNA (tRNA) and ribosomes. tRNA is a specialized RNA molecule that is complementary to mRNA and forms a reversible bond with specific amino acids. The specificity of the amino acid that is added to a tRNA molecule is dependent on the activity of aminoacyl-tRNA synthetase. Aminoacyl-tRNA synthetase joins

together each tRNA to the correct amino acids based on the sequence of the complementary nucleotides at the opposite end of the molecule. Translation starts when the ribosome sequesters a newly synthesized mRNA molecule, and the primary tRNA molecule interacts with ribosome and the mRNA. The primary tRNA binds to the start codon of the mRNA transcript and is held in position by the ribosome. As the mRNA template moves through the ribosome, the next set of trinucleotide codons will interact with the corresponding tRNA. Each time a new tRNA moves into position the protein chain on the outside of the ribosome polymerizes the next amino acid. At the end of translation, the termination signal is recognized and the newly synthesized protein is released from the ribosome.

A fundamental understanding of transcription and translation is vital to advancing the field of forensic serology when it advanced to the molecular base. Forensic serology has focused on the physical and chemical differences between proteins found within forensically relevant body fluids. In keeping with the developing molecular-based trend in forensic science, the products of transcription not translation hold the key to differentiation of body fluids and other forms of human tissue (Crick, 1970).

Types of RNA

Within a cell DNA has a single function, which is to serve as the inheritable molecule. In contrast, RNA molecules have a variety of functions and therefore differ substantially in structure. An example of this variance is tRNA and ribosomal RNA (rRNA). tRNAs are small molecule adapters that convert genetic information, in the form of nucleotides, to structural proteins at the

ribosome. Contained within every ribosome, both eukaryote and prokaryote, is another form of RNA called ribosomal RNA. rRNA acts as a scaffolding which provides physical support for the interaction of transfer and messenger RNAs. tRNA and rRNA exist in all cells and are the same no matter the tissue type. The ubiquitous nature of tRNA and rRNA make them poor candidates for forensic analysis.

Alternatively, mRNA varies based on the tissue type and is produced during transcription. Messenger RNA acts as an intermediate carrier of genetic information between genic DNA and expressed protein, and coordinates with tRNA molecules in order to accomplish translation. Messenger RNA consists of a chain of nucleotides with sequences complementary to the DNA template. The length and nucleotide composition varies between mRNA molecules, which permit the coding of numerous assorted proteins according to different needs of a cell. Although mRNA is crucial, it is not long-lived and is synthesized as needed, so the concentration of mRNA is in flux. In addition, the consortium of mRNA within a given cell varies based on the cell type, making mRNA a viable target for forensic application.

A variety of additional forms of RNA also exist. Small nuclear RNAs (snRNA) possess multiple functions, but most significantly RNA splicing, which is carried out by a large complex called the spliceosome. In most eukaryotic cells, snRNAs form small nuclear ribonucleic protein (snRNP) via the combination of proteins. The main functions of the snRNA with snRNP are to recognize the critical splicing signals at the 5' ends and branch site of introns, combine these sites and ends together, and catalyze the reactions of RNA splicing. Due to the limited number of snRNAs within a cell, they are not ideal candidates for forensic analysis.

Small interfering RNA (siRNA) and microRNA (miRNA) are most often associated with the phenomena of RNA interference (RNAi). RNAi is a biological process in which very small RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNAs. The formation of mature siRNA and miRNA results from the splicing of large RNA precursors. siRNA and miRNA are suited for forensic application because of their small size and sustained stability. In addition, both forms of small RNA are abundant within a cell and vary significantly depending on the tissue type. Table 1 summarizes the type, size and biological function of the most commonly observed RNA in eukaryocyte. The utility of RNA for forensic applications is dependent on degradation rate, size, abundance of the RNA molecule, and whether or not the RNA in question is tissue specific. To date, only mRNA, siRNA, and miRNA satisfy all of these criteria.

Table 1: Type, size and function of RNAs

RNA Type	Size	Function
Messenger RNA	Variable	Directs amino acid sequence of protein synthesis
Transfer RNA	Small	Transports amino acids to site of protein synthesis
Ribosomal RNA	Variable	Combines with proteins to form ribosomes, the site of protein synthesis
Small Nuclear RNA	Small	Processes initial mRNA to its mature form in eukaryotes
Micro RNA	Small	Affects gene expression; important in growth and development
Small Interfering RNA	Small	Affects gene expression; used by scientists to eliminate a gene being studied

RNA Analysis as a Method for the Identification of Forensic Body Fluids

The potential for the identification of body fluids and tissue through the analysis of various types of RNA has not gone unnoticed by the forensic science community (Bauer, 2007). Current

research is actively investigating a number of potential technologies with the desired outcome of discovering a novel procedure for the identification of body fluids that is compatible with current DNA analysis. It has been hypothesized that as genes within tissues are expressed, the RNA profile within each tissue is unique. Assuming a unique RNA signature is possible for all forensically relevant body fluids and tissues, it is logical to conclude that the tissue or body fluid within biological evidence could be uniquely identified.

In 2003, Juusola and Ballantyne published the first in a series of papers on the subject of the forensic application of RNA analysis (Juusola & Ballantyne, 2003). In this paper it was reported that total RNA was successfully isolated from dried blood, semen and saliva stains by acid guanidinium thiocyanate-phenol-chloroform extraction, and quantified using a sensitive fluorescence dye called RiboGreen. Within this publication, Juusola and Ballantyne demonstrated that a sufficient quantity and quality of RNA can be isolated from forensically relevant biological material. In addition, the stability of RNA was also addressed. RNA degrades quickly, is less stable than DNA, and the majority of biological evidence is not collected under ideal conditions. Taken in combination, the application of RNA analysis in forensic casework is dependent on the ability to extract sufficient RNA from the item of evidence. It has been determined by Juusola and Ballantyne that significant quantities of total RNA are recoverable from simulated biological evidence for up to 9 months. In summary, the quantity, quality, and stability of RNA are suitable for forensic applications.

An obvious drawback to RNA analysis is the number of steps and time required to isolate and purify RNA from an evidentiary sample. In addition, the nucleotide sequence homology

between RNA and DNA can cause a substantial complication to any form of analysis if the DNA is not removed from the sample. Therefore, current methods for the extraction of RNA require treatment with DNase I prior to analysis in order to remove DNA contaminants. Current methods for the identification of forensically relevant body fluids are dependent on a reverse transcriptase-polymerase chain reaction (RT-PCR) for the detection of tissue-specific genes within a biological sample. Using the RT-PCR method, the work of Juusola and Ballantyne focused on the unique identification of saliva, because a confirmatory test does not currently exist for this body fluid (Juusola & Ballantyne, 2003). Five "saliva specific" candidate genes were selected based on the fact that their gene expression was restricted to the parotid and salivary glands based on a search of the Cancer Genome Anatomy Project (CGAP) database. The five "saliva specific" candidate genes were: statherin, histatin 3, and the human salivary proline rich proteins PRB1, PRB2, and PRB3. Analysis of the RNA extracted from saliva, blood, and semen supported the author's hypothesis that statherin, histatin 3, PRB1, PRB2, and PRB3 were specific to saliva and therefore not detectable in blood or semen stains.

Expanding on the information obtained from their next series of experiments, Juusola and Ballantyne focused on strengthening mRNA analysis of human body fluids by expanding the number of body fluids analyzed (Juusola & Ballantyne, 2005). The result of their efforts was the creation of a multiplex reaction for the simultaneous analysis of mRNA profiles in an unknown evidentiary sample. Following an exhaustive search of the literature and databases, a set of candidate genes were identified. These genes were selected based on the unique physiology and biochemistry of each forensically relevant body fluid being evaluated. The set of tissue specific

candidate genes were as follows: β-spectrin (blood), porphobilinogen deaminasevz (blood), statherin (saliva) and histatin 3 (saliva), protamine 1 (semen) and protamine 2 (semen), MMP-7 (menstrual blood), human beta-defensin 1 (vaginal secretions), and mucin 4 (vaginal secretions). Two independently regulated genes were selected for each body fluid with the exception of menstrual blood. The selection of two independently expressed systems allowed for the possibility of biological variation in gene expression levels between samples and lessened the probability of false negatives or positives. Analysis of the tissue specific genes listed above produced clear mRNA profiles that corresponded to a single body fluid and yielded no results when other body fluids were analyzed. In addition, the analysis of multiple samples illustrated no false positive or false negative. Moreover, blood from cats, dogs, deer, horses, cattle, sheep, and spider monkeys were all evaluated and each produced negative results. In summary, the mRNA profiling assay developed by Juusola and Ballantyne displayed great potential for application in forensic casework.

By 2007, Juusola and Ballantyne focused his attention on the development of a sensitive and robust method for the analysis of mRNA profiles from forensically relevant body fluids using multiplex real-time quantitative PCR (qRT-PCR). Using qRT-PCR analysis, blood, saliva, semen, vaginal secretions and menstrual blood were evaluated using the same tissue specific genes as before, with the addition of δ-aminolevulinate synthase (blood) and MMP-10 (menstrual blood), and again yielded satisfactory results. Moreover, skin, brain tissue, muscle, adipose tissue, urine and DNA were evaluated for possible false positive, and each yielded the correct negative result (Juusola & Ballantyne, 2007). Of specific interest, were the results obtained from the blood specific genes β-spectrin (SPTB) and porphobilinogen deaminase (PBGD). Both SPTB and PBGD

were detectable in the highest amounts in the blood samples and to a lesser extent in menstrual blood, but were undetectable in saliva, semen, and vaginal secretions stains.

Taken collectively, the research presented by Juusola and Ballantyne established a foundation for an mRNA-based assay for the identification of human body fluids. The potential advantages of this mRNA-based assay include greater specificity compared to conventional methods, the ability to simultaneously analyze multiple markers and tissue types through a common assay format, and the possibility of a semi-automated technique. The use of the qRT-PCR process is still not without complications. For example, using qRT-PCR in forensic casework samples suggests that the process will result in the addition of multiple steps to existing protocols, which would increase the amount of time per case. In addition, current forensic serology tests aid in the discovery of suitable biological stains to analyze, and the qRT-PCR technique described above is not suitable for such a function. If a method were developed that could directly detect tissue specific mRNA, then mRNA based methods for body fluid identification would be fast enough for accurately screening biological evidence prior to DNA analysis.

Hypothesis for the Proposed Thesis

Forensic serology tests can help determine the type of biological material found on an item of evidence; however, there are a limited number of confirmatory tests currently available. While DNA analysis can help identify the contributor of a given biological material, it cannot establish from what tissue the DNA originated. On the other hand, some RNA molecules are found to be tissue specific, and the detection of specific tissue mRNAs is currently possible. However, the

current method of choice for the analysis of mRNA is qRT-PCR, which is not compatible with the needs of the forensic analysts screening the evidence. An alternative method for the analysis of mRNA that is independent of PCR amplification may exist. It is well established that complementary nucleic acids can bind to each other in a highly specific manner. The hybridization of a nucleotide probe to a specific mRNA can form a double stranded nucleic acid strand that can then be labeled to indicate a positive result. Therefore, it is hypothesized that the mRNA transcripts from the blood specific genes β-spectrin (SPTB) and porphobilinogen deaminase (PBGD) can be detected through hybridization with a complementary probe.

Material and Method

The primary goal of this thesis was to determine if the hybridization of an oligonucleotide probe to a known tissue specific mRNA could be detected independent of amplification. It was quickly determined that the repeated use of the same extracted RNA samples was resulting in the loss of the RNA, and an alternative approach was developed. As with other studies, the mRNA transcript of β-spectrin and porphobilinogen deaminase were converted into their complementary DNA and amplified via reverse transcriptase PCR. Contrary to other methods, the resulting products from the amplification were used for hybridization assays. It was assumed that upon successful detection of probe-cDNA hybrids the method would be reproduced using probe-mRNA hybrids.

Tissue Specific Primers Design

DNA primers for the human housekeeping genes S15, GAPDH and β-actin and blood specific genes SPTB and PBGD were purchased from Integrated-DNA Technologies®. The primer sequences illustrated in Table 2 were selected from the work of Juusola and Ballantyne (2003, 2005 and 2008). Juusola and Ballantyne's findings demonstrated the viability of each primer set for forensic application. In addition, the decision was made to use DNA oligonucleotide rather than RNA oligonucleotide for the hybridization analysis due to the tendency of RNA to form secondary structures. Oligonucleotide primers arrived freeze-dried and were resuspended in 1×TE buffer to obtain a 100µM freezer stock, and then diluted to a 10µM working stock and 3.2 µM

sequencing stock.

Extraction and Quantitation of RNA from Human Blood

Table 2: Properties of Primers

Name	Sequence (5' to 3')	Bases	T_m (°C)	GC %	Type
S15-(F)	TTCCGCAAGTTCACCTACC	19	54.9	52.6	Housekeeping
S15-(R)	CGGGCCGGCCATGCTTTACG	20	64.3	70	Housekeeping
ß-actin-(F)	TGACGGGGTCACCCACACTGTGCCCATCTA	30	68.6	60	Housekeeping
ß-actin-(R)	CTAGAAGCATTTGCGGTGGACGATGGAGGG	30	65.2	56.6	Housekeeping
GAPDH-(F)	CCACCCATGGCAAATTCCATGGCA	24	62.6	54.1	Housekeeping
GAPDH-(R)	TCTAGACGGCAGGTCAGGTCCACC	24	63.9	62.5	Housekeeping
SPTB-(F)	AGGATGGCTTGGCCTTTAAT	20	54.5	45	Blood
SPTB-(R)	ACTGCCAGCACCTTCATCTT	20	56.9	50	Blood
PBGD-(F)	TGGATCCCTGAGGAGGGCAGAAG	23	62.4	60.8	Blood
PBGD-(R)	TCTTGTCCCCTGTGGTGGACATAGCAAT	28	63.1	50	Blood

Note. Information from Integrated DNA Technologies®, including the Name, Sequence, Bases, Tm(°C), GC Content (%) and Type of each primer ordered.

In accordance with the University of Central Oklahoma's Institutional Review Board, human whole blood, preserved with ethylene diamine tetraacetic acid (EDTA), was purchased from Innovative Research™. In order to obtain RNA samples suitable for downstream analysis the Qiagen QIAamp® RNA Blood Mini Kit was utilized (Qiagen, 2010). A total of twenty replicates were extracted and purified using the following protocol. One milliliter of human whole blood was suspended in 5 mL of Buffer EL, and incubated on ice for 15 min. Following incubation, all samples were centrifuged at 400×g and 4°C for 10 min. The supernatant was discarded and 2 mL of Buffer EL were again added to each sample, followed by centrifugation at 400×g and 4°C for 10 min. The supernatant was disposed of once more and 600µl of Buffer RLT was added to each

tube followed by centrifugation. The lysate was subsequently pipetted into a QIAshredder spin column nested in a 2 ml collection tube and centrifuged for 2 min at maximum speed. The QIAshredder spin column was discarded and 600µl of 70% ethanol was added and mixed to the lysate. The entire sample was transferred into a new QIAamp spin column and centrifuged for 15sec at 8000×g. The resulting flow-through and the collection tube were disposed of, and again the spin column was transferred into a new 2 mL collection tube. The column was washed with 700 µL of Buffer RW1 and centrifuged at 8000×g for 15sec. The column was washed two more times with 500 µL Buffer RPE, and centrifuged at 8000× g for 15 sec and full speed (20,000×g) for 3min respectively. The totality of the flow-through was discarded, and the spin column and collection tube were centrifuged at full speed for 1 min to eliminate the possibility of carryover from Buffer RPE. Finally, the QIAamp spin column was transferred to a new 1.5 mL microcentrifuge tube, and 50 µL of RNase-free water was added directly onto the QIAamp membrane. The 1.5 mL microcentrifuge tube was centrifuged for 1 min at 8000× g and the purified RNA was eluted into the 50 µL of RNase-free water.

Following extraction and purification each sample was quantified using a Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo Scientific, 2009). Each sample was quantified for the total amount of dsDNA, RNA and ssDNA prior to DNase I treatment. Before the collection of measurements, RNase-free water was used to blank the instrument, and between each sample a dry, lint-free laboratory wipe was used to wipe away the liquid.

Digest DNA with DNase I

Promega RQ1 RNase-Free DNase (Promega, 2009) was applied to eliminate the DNA from extracted and purified samples. DNase treatment was performed in order to improve the quality of

the RNA within the previously extracted samples, and to avoid the possibility of cross-hybridization from genomic DNA. 1mL of 10× Reaction Buffer was added to 1 μL of 1U/μL DNase and 8μL of RNA. Each sample was incubated at 37℃ for 30min, followed by the addition of 1μL of RQ1 DNase Stop Solution and an additional incubation of 10min at 65℃ to inactivate the DNase. The concentration of dsDNA, RNA and ssDNA for each sample was again measured following DNase treatment using a NanoDrop 2000 Spectrophotometer to verify the efficiency of DNase.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Reverse transcriptase PCR was employed in order to convert the desired mRNA transcripts into complementary DNA. Complementary DNA was then amplified in order to obtain a suitable amount of genetic material by which to perform all subsequent experiments. The SuperScript™ One-Step RT-PCR with Platinum® Taq System (Invitrogen, 2010) was utilized as a one-step solution for the amplification of cDNA from an mRNA starting product. All reagents from the RT-PCR System were placed on ice, and RNA samples and primers were allowed to melt at room temperature. All reactions took place in a 0.2 mL PCR tube, and each primer pair listed in Table 2 was tested. To each PCR tube the following was added: 25μl of 2× Reaction Mix, 1μL template RNA (10pg – 1μg), 1μL forward primer (10nM), 1μL reverse primer (10nM), 1μL RT/ Platinum® Taq Mix and water to total 50μL. Each reaction tube was gently mixed, and a GeneAmp® PCR System 9700 thermal cycler was used to perform the RT-PCR process. The thermal cycling profile for the RT-PCR was divided into two steps which ran in tandem. The conversion of RNA into

cDNA was achieved by incubation at 50°C for 30 min. followed by 94°C for 2 min. Amplification of cDNA was obtained by performing 35 cycles of 94°C for 15sec, 55°C for 30sec, and 72°C for 1 min. A final extension step was performed at 72ºC for 10 min. and then stored at 4 ºC until needed. A reagent blank control was also performed in order to identify the possibility of contamination.

Confirmation of Target Gene Specificity by Cycle Sequencing

In order to verify the specificity of the primer pairs for each gene of interest, cycle sequencing was performed. PCR buffer optimization was performed using the FailSafe™ PCR PreMix Buffer system (Epicentre, 2013). The FailSafe™ PCR PreMix Buffer system consists of twelve buffers labeled A through L, and each buffer contains dNTPs and varies in pH and MgCl concentration. All twelve buffers were evaluated while all other running conditions remained consistent. Each reaction contained the following: 12µM forward and reverse primers, 10ng of cDNA template and 1U of AmpliTaq Gold® Polymerase, 3.5µL of FailSafe™ PCR PreMix Buffer A-L and 12.9µl water. The reagent blank control was performed using the S-15 primer set and 1.0µL water in place of cDNA. Amplification was performed in a GeneAmp® PCR System 9700 thermal cycler using the following run conditions: 98°C for 2 min. followed by 40 cycles of 98°C for 10sec, 50°C for 30sec, and 72°C for 1 min. Final extension was performed at 72ºC for 7 min. and samples were stored at 4ºC. The resulting PCR products were analyzed by electrophoresis using a 1% agarose gel and stained with ethidium bromide. Electrophoresis was performed at 60V for 90 min., and results were imaged using a Gel Logic 100 Imaging System.

Following agarose gel electrophoresis, all samples that presented a band were purified using the Qiagen QIAquick® PCR Purification Kit (Qiagen, 2008). For the QIAGEN QIAquick® PCR Purification Kit, 100 µL of Buffer PB was added to each PCR sample, and allowed to come to equilibrium. The 120µL sample was then applied directly on to the QIAquick spin column and centrifuged for 60s at 17,900×g in room temperature. The flow-through was discarded and 750µl of Buffer PE was added to the QIAquick column. Again the entire column was centrifuged for 60s and the flow-through discarded. The residual ethanol was completely removed with an additional 1 min centrifugation, and 50 µl Buffer EB (10mM Tris•Cl, pH 8.5) was added to elute DNA from the column. This time the flow-through was collected in a clean 2 mL microcentrifuge tube.

The BigDye® Terminator v3.1 Cycle Sequencing system (Applied Biosystems, 2010) was employed to obtain the nucleotide sequence for the purified PCR products obtained from the previous step. In accordance with the manufacture's protocol, each cycle sequencing reaction consisted of 8.0µl of terminator ready reaction mix, 3.0µl of purified PCR product, 3.3pmol of either the forward or reveres primer, and 8.0µl of water. As before, both positive and negative control and reagent blank were performed. Amplification was performed in a GeneAmp® PCR System 9700 thermal cycler using the following thermal cycling profile: 96°C for 1 min.; followed by 25 cycles of 96°C for 10sec, 50°C for 5sec and 60°C for 4 min. cycle sequencing product which remained at 4^{o}C until needed.

Cycle sequencing product was purified prior to capillary electrophoresis using PERFORMA® DTR Gel Filtration Cartridges (EdgeBio, 2013). Centrifugation of the filtration cartridge was performed for 3 minutes at 850×g (3300rpm). The filtration cartridge was then

transferred to a new 1.5 mL microcentrifuge tube and the sample was added to the top of the packed column. The sequencing product was centrifuged for 3 minutes at 850×g (3300rpm) and the resulting flow-through was kept for downstream analysis.

Fragment analysis of cycle sequencing products was performed on an ABI 3130 Genetic Analyzer using POP-4™ polymer and a 36 cm capillary. In a 96 well plate, 10 μL of Hi-Di formamide was added to 10 μL of each sample to be analyzed. Sequencing analysis was performed on a single cDNA sample obtained from human whole blood using five sets of primers. The samples analyzed were labeled as follows: DNA control β-act-reverse, DNA control β-act-forward, S15 reverse, S15 forward, β-act-reverse, β-act-forward, SPTB reverse, SPTB forward, SPTB-reverse, SPTB-forward, P-reverse, P-forward, G-reverse, G-forward, Standard DNA (positive control), Blank β-act-reverse and Blank β-act-forward. Sequence data analysis was performed using Sequencher 5.1 software and searched for similar sequences using the National Center for Biotechnology Information Blast search engine.

Dot Hybridization of cDNA to Oligonucleotide Probe

To construct the blotting manifold, a section of Millipore Immobilon™-Ny+ Transfer membrane was cut to the designed size, and labeled with a soft pencil to indicate the proper orientation. The membrane was briefly placed in RNA-free water then soaked in 20× SSC solution (3M NaCl, 300mM sodium citrate, pH7) for 1h at room temperature. The blotting manifold was cleaned with 0.1N NaOH, and rinsed with RNA-free water prior to use. A stack of paper towels, which functioned as blotting paper, was prepared, and the top two sheets were wet with 20× SSC.

The nylon membrane was then placed on the bottom of the plate, air bubbles were removed, and the paper towels were placed on top of the nylon membrane. Each dot was then allowed to absorb 10× SSC twice in order to stimulate nucleotide binding.

Products for the blood specific gene SPTB, housekeeping gene S15 and "blank" as the reagent control from the reverse amplification performed previously were selected as the samples needing to be detected. RNA-free water functioned as a negative control. 1μL of each sample was dissolved in 9μL of RNA-free water, mixed with 30μL of RNA denaturation solution (600 μL formamide, 210μL formaldehyde (40%, v/v) and 130μL 10× MOPS electrophoresis buffer, pH 7.0), incubated for 5min at 65℃, and allowed to cool on ice. The samples were then transferred and fixed to the membrane. Following the second administration of 10× SSC to each of the test sites, 40μL of the samples was loaded on to each test site and allowed to pass through the membrane. Following the transfer of the nucleic acid onto the membrane, the membrane was rinsed twice with a large amount of 10× SSC and then dried for about 10min. The membrane was then placed on a dry paper towel and placed into a SPECTROLINKER^{TM} XL-1500 UV Crosslinker. Samples were cross-linked to the membrane by UV irradiatde at 254nm.

The final step in hybridization protocol was the hybridization of the oligonucleotide probes to the immobilized sample. The membrane containing the immobilized samples was incubated with 10-20mL of prehybridization solution (1 mM EDTA, pH 8, 0.5M Na_2HPO_4, pH 7.2, 7% SDS) in a 15mL conical screw cap tube for 2h at 68^{o}C in a water bath. After incubation, the 15μL of denatured probe was added directly into the prehybridization solution, and allowed to incubate 17hrs at 68^{o}C in a water bath. After hybridization, the membrane was removed from the 15mL

conical screw cap tube, and transferred to an empty sterilized plastic tip box containing 100-200mL of 1× SSC and 0.1% SDS solution at room temperature. The closed box was agitated on a platform shaker for 10min. The membrane was again transferred to another empty sterilized plastic tip box containing 100-200mL of 0.5× SSC and 0.1% SDS solution prewarmed to 68^{o}C, and agitated for another 10min. The washing step was performed twice. The membrane was placed in a baking dish with 200 mL of 0.1% SDS solution and 10μL ethidium bromide, and allowed to incubate at room temperature for 2min. Finally, the membrane was imaged using a Gel Logic 100 Imaging System.

Results

Extraction, Quantification, and Amplification of RNA from Human Blood

Utilizing the Qiagen QIAamp® RNA Blood Mini Kit, total RNA from human whole blood was successfully extracted. In total, twenty replicates were extracted, and eighteen extracts had adequate concentration of RNA for subsequent analyses. RNA concentration was determined by UV spectrophotometry, using a NanoDrop 2000 spectrophotometer. The average RNA concentration for the eighteen samples used for downstream analysis was greater than 1000ng/µL. In addition, RNA purity was assessed by 260/280 ratio, and the majority of samples were of sufficient purity following DNase treatment.

Primer pairs for the amplification of blood specific and housekeeping mRNA genes (Table 1) were utilized for the conversion of mRNA to DNA followed by PCR amplification. Using the SuperScript™ One-Step RT-PCR with Platinum® Taq System, the five primer sets, labeled as β-actin, GAPDH, PBGD, S15, and SPTB, were amplified under sterile laboratory conditions. In addition, a reagent control labeled as “Neg Con” and a DNA control were amplified in order to evaluate the experiment for contamination. The reagent control originated at extraction and was performed in parallel, but lacked any form of biological material. The DNA control lacked the reverse transcriptase enzyme necessary to convert RNA into DNA and would therefore function to evaluate the possibility of DNA contamination. As can be seen in Figure 5, the control samples, “Neg Con” and “DNA Con”, failed to produce a band. The lack of an amplification product provided indications that contamination was not encountered. The PCR product from the β-actin,

GAPDH, and S15 reactions produced bright, robust bands and the approximate size was consistent with what was predicted. The SPTB PCR product was observed at a notably reduced concentration, while the product from the PBGD reaction was entirely absent. The difference in band intensity between the three housekeeping genes and SPTB is most likely due to the starting concentration of each mRNA transcript. The majority of "housekeeping" genes perform an essential function and are therefore in high concentration within the cell. In summary, the RT-PCR reaction for the primer sets β-actin, GAPDH, and S15 yielded sufficient quantities of product, and no contamination was detected.

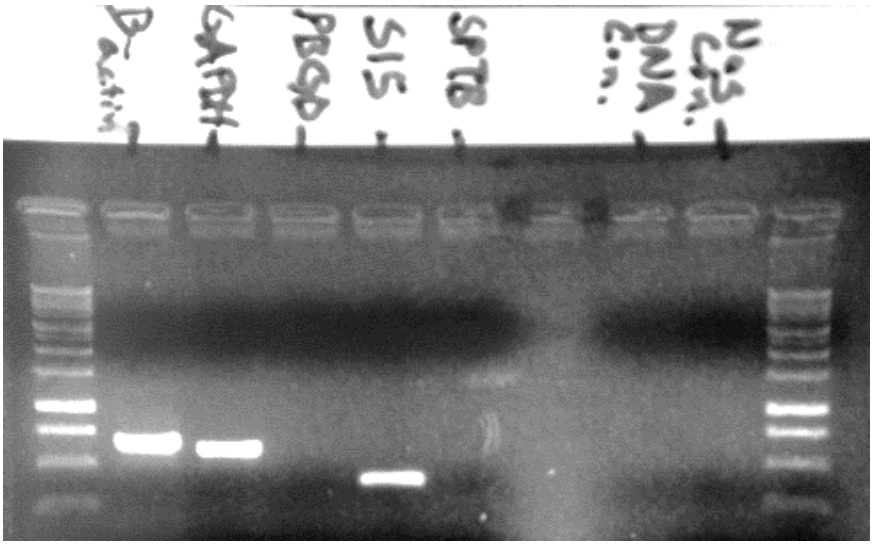

Figure 5. RT-PCR amplification of housekeeping and blood specific genes.

Confirmation of target gene specificity by cycle sequencing

cDNA from samples β-actin, GAPDH, SPTB and S15 were sequenced to confirm the specificity of each primer set. Utilizing the BigDye® Terminator v3.1 Cycle Sequencing system, 10μL of cycle sequencing product was analyzed. The resulting sequences were then evaluated for

errors and trimmed. Trimming the sequence data was done in order to remove the artifacts frequently observed at the beginning and end of the electropherogram. The ClustalW - Multiple Sequence Alignment tool was used in order to align the multiple reads, and a consensus sequence was constructed for each of the four samples. The results for the sequencing, after trimming, were as follows:

Consensus sequence for β-actin cDNA.

GGTTGCCCTCCCCATGCCATCCTGCGTCTGGACCTGGCTGGCCGGGACTGACTGT
ACTACCTCATGAAGATCCTCACCGAGCGCGGCTACAGCTTCACCACCACGGCCGA
GCGGGAAATCGTGCGTGACATTAAGGAGAAGCTGTGCTACGTCGCCCTGGACTTC
GAGCAAGAGATGGCCACGGCTGCTTCCAGCTCCTCCCTGGAGAAGAGCTACGAG
CTGCCTGACGGCCAGGTCATCACCATTGGCAATGAGCGGTTCCGCTGCCCTGAGG
CACTCTTCCAGCCTTCCTTCCTGGGCATGGAGTCCTGTGGCATCCACGAAACTACC
TTCAACTCCATCATGAAGTGTGACGTGGACATCCGCAAAGACCTGTACGCCAACA
CAGTGCTGTCTGGCGGCACCACCATGTACCCTGGCATTGCCGACAGGATGCANAA
AGGAGATCACTGCCCTGGCACCCAGCACAATGAAGATCAAGATCATTGCTCCTCC
TGAGCGCAAGTACTCNGTGTGGATCGGCGGCTNCATCNTGGCCTCGCTGTCCACN
TTCCAGC

Consensus sequence for GAPDH cDNA.

CATTGGCGTGGGGNCGGAAGGCCATGCCAGTGAGCTTCCCGTTCAGCTCAGGGTA
TGACCTTGCCCACAGCCTTGGCAGCGCCAGTAGAGGCAGGGATGATGTTCTGGAG
AGCCCCGCGGCCATCACGCCACAGTTTCCCGGAGGGGCCATCCACAGTCTTCTGG
GTGGCAGTGATGGCATGGACTGTGGTCATGAGTCCTTCCACGATACCAAAGTTGT

CATGGATGACCTTGGCCAGGGGTGCTAAGCAGTTGGTGGTGCAGGAGGCATTGCT
GATGATCTTGAGGCTGTTGTCATACTTCTCATGGTTCACACCCATGACGAACATGG
GGGCATCAGCAGAGGGGGCAGAGATGATGACCCTTTTGGCTCCCCCCTGCAAATG
AGCCCCAGCCTTCTCCATGGTGGTGAAGACGCCAGTGGACTCCACGACGTACTCA
GCGCCAGCATCGCCCCCCTTGATTTTGGGAGGGATCTCGCTCCTGGAAGANGGTG
ATGGGATTTCCATTGATGAAAAGCTTNCCGTTCTCAGCCTTGACGGTGCCATGNAA
TTGGCC

Consensus sequence for S15 cDNA.

CTCNCAGCTGCTGGAATGTCCTANGAGCAGCTGATGCAGCTGTACAGTGCGCGCC
AGCTGCGGCGGCTGAAACCGGGGCCTGCGGCGGAGCAGCACTCCCTGCTGAAGC
GCCTGCGCAAGGCCAAGAAGGAGGCGCCGCCCATGGAGAAGCCGGAAGTGGTG
AAGACGCACCTGCGGGACATGATCATCCTACCCGAGATGGTGGGCAGCATGGTGG
GCGTCTACAACGGCAAGACCTTCAACCAGGTGGAGATCAAGCCCGAGATGATCG
GCCACTACCTGGGCGAGTTCTCCATCACCTACAAGCCC

Consensus sequence for SPTB cDNA.

GNCCGGCCGACCTGATCGACTTTGATAAGCTGAAGGACTCCANTGCCCGGCACNT
TCNCCCAGCACGCGNGGGGAANAAGNNTGNAGCGCCAGCTGGGCATCATCCCGC
TCCTCGACCCCGAAGATGTCTTTACGGAAAACCCTGATGAGAAATCCATCATCACC
TATGTGGTGGCCTTTTACCACTACTTCTCCAA

Following the construction of each consensus sequence, all four sequences were evaluated

for sequence homology with previously published data by submission to the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database via the Basic Local Alignment Search Tool (BLAST). As was expected, all four sequences returned the corresponding result. Using the consensus sequences for β-actin, GAPDH, S15, and SPTB cDNA, a GEO search was reported *Homo sapiens* mRNA genes beta actin (NM_001101.3), GAPDH (NM_002046.4), ribosomal protein S15 (BC141832.1), and erythrocytic beta spectrin (NM_000347.5) respectively. In all cases the query cover, E value, and percent identity were all acceptable, with the weakest homology being displayed by SPTB with an E value of 1e-64 and percent identity of 89%. All four primer sets demonstrated the ability to specifically amplify the human genes reported previously. Therefore, the RT-PCR product isolated from each reaction was suitable for all subsequent experiments.

Dot hybridization of cDNA to oligonucleotide probe

Essential to the success of this project was an understanding of the physical properties of the Millipore Immobilon^{TM}-Ny^{+} transfer membrane. In order to characterize a few of these properties, the Millipore Immobilon^{TM}-Ny^{+} transfer membrane was subjected to a simple diffusion test. Figure 6 depicts the results of the diffusion test. As can be seen in Figure 6, the mobility of the substrate on the transfer membrane was not consistent between wet and dry studies. As was expected, the use of a wet membrane increased the diffusion range of the substrate and could lead to the cross-contamination of adjacent samples.

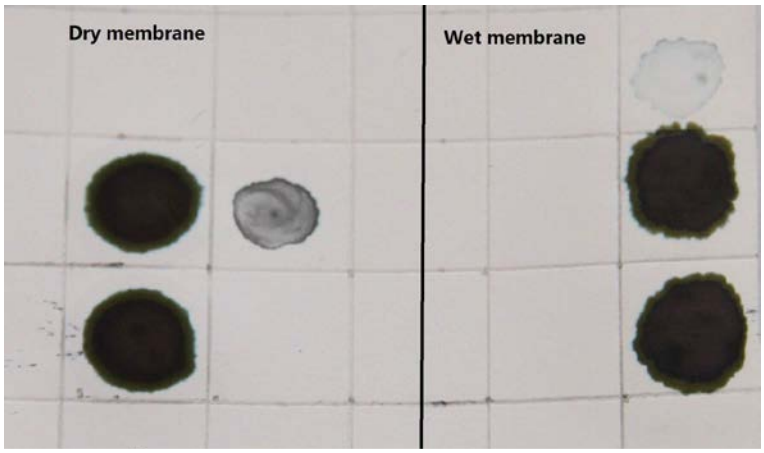

Figure 6. Testing the diffusion of the membrane. This figure illustrates the difference in diffusion rate for dry and wet membranes.

Figures 7 through 10 are an illustration of the results obtained from evaluating the transfer membrane's ability to bind and hold charged substrates like DNA, RNA, and protein. In this experiment, a visually identifiable charged protein was added to the membranes, both wet and dry (Figure 7). The substrate was bound to the transfer membrane by UV irradiated (Figure 8). The transfer membrane was washed twice with 1x SDS buffer in an attempt to remove the majority of nonspecific binding (Figure 9). Finally, the transfer membrane was washed with 1× SDS buffer (Figure 10). After each step the membrane was imaged and evaluated for loss of color intensity and substrate diffusion. This series of tests depicted in figures 7 through 10, indicated that the protein substrate remained bound to the membrane under all conditions tested. Therefore, the hybridization protocol was determined to be adequate for the analysis of cDNA obtained from the RT-PCR reactions performed previously.

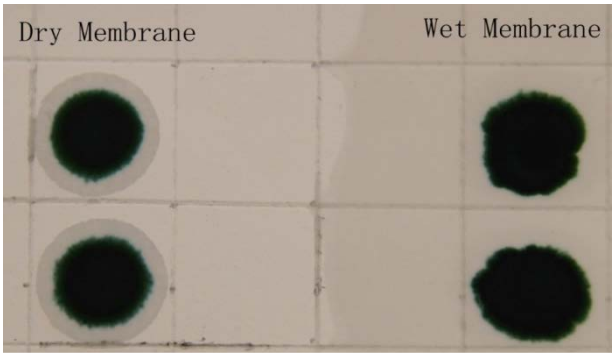

Figure 7. Binding ability of the membrane, step one. This figure illustrates the process of apply 10μL loading dye in different area of the membrane, after right half of the membrane was moisturized with RNA free water.

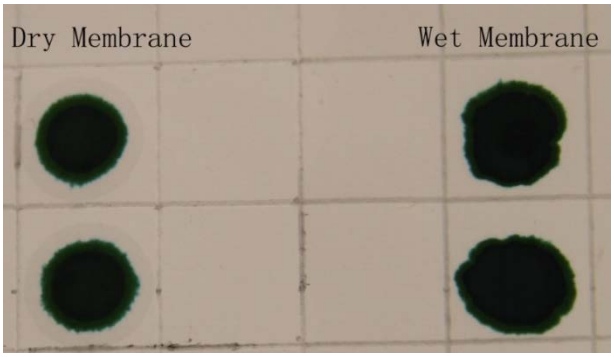

Figure 8. Binding ability of the membrane, step two. This figure illustrates the process of irradiate the membrane at 254nm, 1.5J/cm^2 (that is 15×100μJ/cm^2) in the SPECTROLINKER™ XL-1500 UV Crosslinker, to cross-link the membrane.

The final evaluation of the Millipore Immobilon™-Ny$^+$ transfer membrane was conducted

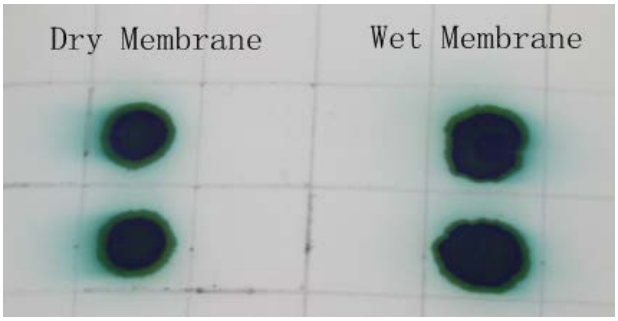

Figure 9. Binding ability of the membrane, step three. This figure illustrates the process of washing the membrane twice with 1× SDS buffer for 15min each at room temperature.

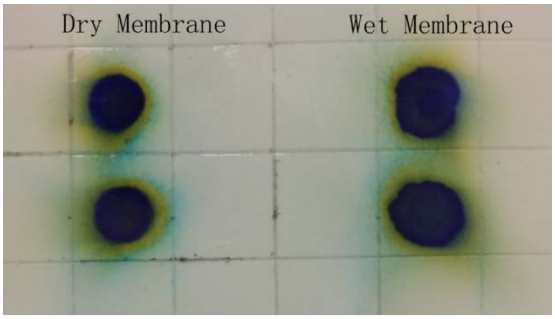

Figure 10. Binding ability of the membrane, step four. This figure illustrates the process of the final wash for 30min at 55℃ in 1× SDS buffer.

in order to determine the amount of background signal emitted by the membrane. In this experiment, ethidium bromide was added to the final wash step and allowed to soak for 2 min at room temperature. Following incubation the membrane was evaluated for nonspecific fluorescence. It was determined that the amount and random pattern of background fluorescence was less than ideal, but did not present an insurmountable challenge (Figure 11 and 12).

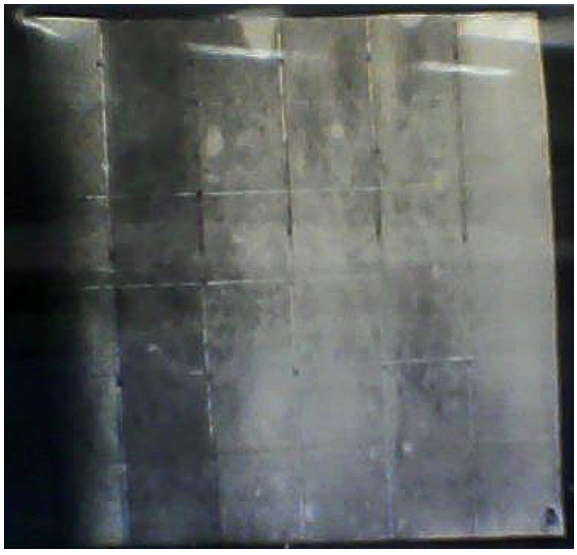

Figure 11. Testing the background signal of the wet membrane. This figure illustrates the background signal when membrane is wet.

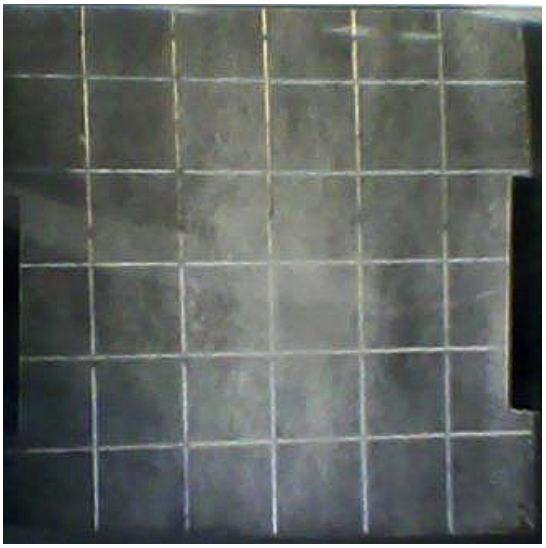

Figure 12. Testing the background signal of the dry membrane. This figure illustrates the background signal when membrane is dry.

For the hybridization of cDNA to the oligonucleotide probes experiment, the Millipore Immobilon™-Ny+ transfer membrane was designed in a1cm×1cm grid pattern. Each probe was placed on the membrane in duplicate along with the extraction blank and negative controls consisting of water (see Figure 13).

S	S15	G	b	S
b	Neg Cont	S	Neg Cont	S15
G	b	Blank	S15	G
S15	Neg Cont	G	Neg Cont	b
S	b	S	S15	G

Figure 13. Membrane designed without primer P. This figure illustrates the design of membrane without primer P for the primers efficiency test.

In order to test the effectiveness of RNA samples cross-linked to the nylon membrane, two sets of dot hybridization assays for the RT-PCR products were conducted on two consecutive days. One set for the mix samples (Figure 14) and the other (Figure 15) is for single sample identification. "S" is the RT-PCR product for the blood specific gene SPTB, "S15" is the RT-PCR product for the housekeeping gene S15, "Blank" is the reagent control which contained no biological material, and "Neg" is merely RNA-free water, "Blank" and “Neg” served as the contamination indicators.

As can be seen in Figures 14 and 15, all data obtained were indistinguishable from the background. The results of this experiment indicate that the hybridization assay used in this experiment was not sensitive or specific enough to detect the small amounts of cDNA present in these samples.

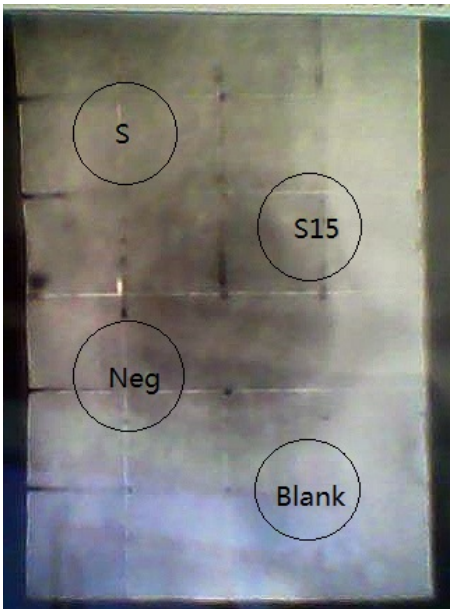

Figure 14. Mix dot hybridization. This figure illustrates the result for the mix dot hybridization of sample “S15 5”, “S 2”, “Bank” and Negative control. No positive result showed up.

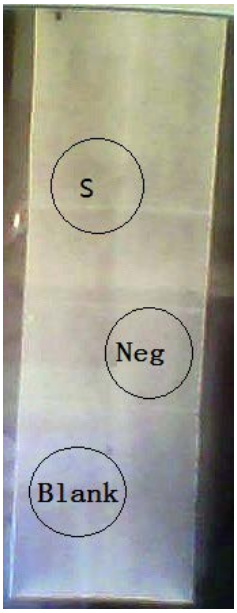

Figure 15. Single dot hybridization. This figure illustrates the result for the single dot hybridization of sample “S 2”, “Bank” and Negative control. No positive result showed up.

Discussion

Current methods for the analysis and identification of forensically relevant body fluids utilize PCR amplification, techniques that are not compatible with forensic case workflow. The trend within the forensic science community is driving toward reducing the time necessary to process a case, and the use of amplification-based techniques increase the time and expense of each case. To better suit the needs of the forensic community, the research approach of this thesis focuses on investigating the utility of mRNA hybridization for the identification of human blood.

The goal of this research project was to apply a non-PCR based analysis method for the identification of mRNA specific to human blood. In principle, DNA probes specific to human blood genes would be rendered stationary by UV cross-linking to a nylon membrane. RNA from a human blood sample would be extracted and incubated with the nylon membrane. The affinity between the total RNA sample and the complementary DNA probes would hybridize the two of them together and form an artificial double-stranded nucleic acid. The resulting hybridization between the newly formed DNA-RNA complex would then be treated with ethidium bromide, which would function as an indicator that could be detected under ultraviolet light.

The development of a rapid screening method like that described above is essential to the advancement of forensic science. In our experience, the majority of the primers tested amplified the expected target gene; however, the use of cDNA samples cross-linked to the nylon membrane demonstrated minimal success. The administered sample rapidly defused radially, and a substantial amount of background noise existed when imaged. Modification of the protocols continued to

produce similar results. Therefore, the hypothesis outlined for this thesis was conclusively rejected. While the hybridization of cDNA and DNA was effective, the ability to accurately detect the abundance of these duplexed molecules was inadequate. The inadequacy of the detection method can most likely be contributed to the sensitivity and specificity of ethidium bromide. It is therefore recommended that an alternative detection method be investigated.

According to the presentation, *The Changing Face of Body Fluid Identification*, as presented by Sally Ann Harbison (2013) at the 25th World Congress of the International Society for Forensic Genetics, the most advanced techniques available today are still not specific enough for the definitive identification of body fluids. Therefore, further studies are necessary, and a more effective and user-friendly mRNA assay for the identification of forensically relevant body fluids is integral to this effort. With this in mind, the use of Fluorescence Resonance Energy Transfer (FRET) probes was identified as the most logical alternative approach to the outlined hypothesis. The use of FRET probes is a promising advance that could potentially provide the increased sensitivity necessary to detect the mRNA-DNA hybrid.

According to *An Introduction to Fluorescence Resonance Energy Transfer (FRET) Technology and its Application in Bioscience* (Held, 2005), FRET is a physical phenomenon of the radiationless transmission from the excited state energy of the originally excited donor to an acceptor. The donor molecule is the dye that initially absorbs the energy, and the acceptor is the dye to which the energy is subsequently transferred. Without conversion to thermal energy and without contact between the two molecules, FRET leads to a reduction in the donor's fluorescence strength and excited state lifetime, and to an increase in the acceptor's emission intensity. FRET is

distance-dependent and extremely sensitive over small distances. The measurement of FRET efficiency between two probes can be used to determine the presence or absence of a nucleic acid hybrid. If the possibility exists for the unique identification of a human body fluid based on the principles of hybridization, then FRET analysis is ideally suited for such an assay.

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