FORENSIC STAIN IDENTIFICATION BY RT-PCR ANALYSIS

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ABSTRACT

With the advent of innovative molecular biological techniques becoming the norm in the forensic laboratory, it is plausible to imagine the eventual replacement of the serological testing methods traditionally used to identify questioned stains with molecular biological techniques. New tests that are tissue specific and designed to be multiplexed would yield rapid results on a minimal amount of sample. Such testing could employ mRNA as the tissue-specific determinant by first identifying and then testing for the appropriate tissue-specific mRNAs. Analyses can also be performed to demonstrate that mRNA is relatively stable and can thus be of great use in a wide variety of forensic cases. The nature of this research was to identify mRNA transcripts that will definitively identify the tissue of origin, determine if such transcripts survive the typical environmental insults that forensic samples may encounter, and develop rapid assays using small amounts of sample. Initial work focused on developing a method to co-extract DNA and RNA from the same sample. Several commercial methods were compared for the ability to isolate high quality nucleic acids. Through these studies we optimized a DNA extraction method which has become our standard procedure for extraction of CODIS samples. We evaluated the stability of RNA over time using real-time TaqMan®-based PCR assays for the detection of blood- and semen-specific genes. Once RNA was shown to be stable in samples aged up to 4 years, we sought to identify 2-3 tissue-specific transcripts for a variety of stains. In addition to specificity,

the sensitivity of the assays was also determined using different sized stains. Several technologies were used to multiplex assays once candidates were shown to be tissue-specific. The Plexor® One-Step qRT-PCR System was used to develop three different multiplex assays: blood-semen, semen-sperm and semen-saliva. The semen-saliva assay was designed in collaboration with Promega and is currently entering the final stages of development. Through the work with Promega, a RNA/DNA co-isolation technique was developed which effectively extracts both nucleic acids of sufficient quality and quantity for downstream real-time PCR and STR analyses. Homebrew TaqMan® assays were developed for semen-sperm identification as well as a brain screening assay. Lastly, Luminex-bead technology in conjunction with the QuantiGene® Plex Reagent System was evaluated for its ability to detect tissue-specific markers in forensic stains. Importantly, this system does not require RNA extraction which increases the efficiency of the assay in addition to offering another platform for multiplex analysis.

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EXECUTIVE SUMMARY

Identification of the tissue origin of the suspect DNA is often an important issue in forensic casework, which may also aid in predicting the success of DNA analysis. Therefore, it is paramount to the criminal justice system that suspect stains be identified definitively and accurately. Currently, the serological approaches for stain identification involve enzymatic or immunologic tests. While these tests have improved in selectivity over the years, several problems still exist such as the possibility of cross-reactivity with other species and the lack of specificity for particular tissues. In addition, there are several tissues for which no such tests are available.

New tests that are fluid/tissue-specific and designed to be multiplexed would yield rapid results on a minimal amount of sample. Such testing could employ mRNA as the specific determinant. While the DNA of all tissues from an individual is essentially identical, the mRNA spectrum made by the different cells in each tissue is very different. Each tissue or cell type makes a unique constellation of mRNAs, some specific for only that tissue or cell type. Some body fluids, such as blood, contain cells as part of their function while other fluids, such as urine, contain cells that have been shed from their tissue of origin. Therefore, analysis of the "RNA profile" in a sample can uniquely identify the fluid or tissue of origin.

As the demand for sample analysis increases, forensic laboratories continue to balance manpower and cost issues versus the value of the analysis when evaluating new techniques. In order for laboratories to invest in RNA technology, they will require a straight-forward extraction procedure. A method that could co-purify RNA and DNA from a single sample with a

minimal number of steps would be attractive to those seeking new technologies. A preferred extraction method would demonstrate the best ability to co-isolate DNA and RNA in terms of yield and amplifiability while remaining simple, efficient, and ideally, involve non-hazardous reagents.

The thrust of this research was to identify mRNA transcripts that will definitively identify the tissue of origin, determine if such transcripts survive the typical environmental insults that forensic samples may encounter, and to develop rapid multiplex assays to assess these molecules using small amounts of sample. The proposal had the following goals: 1) to select the best commercial kit to extract mRNA from a wide variety of stain types, 2) to determine the half-life of mRNA in different types of stains under different conditions, 3) to find 2-3 genes specific for each of four stain types (semen, blood, urine, saliva), 4) to develop multiplex assays for the identification of stain types, 5) to validate these assays for casework samples, and 6) to disseminate these results to the forensic community.

Our first goal was to identify the best method to extract RNA from a variety of stain types. Throughout the course of the grant, we tried several commercial extraction kits which had various degrees of success. In addition, we sought a protocol which would allow the simultaneous or co-extraction of DNA. Parallel stains were made with a measured amount of blood or semen and RNA isolated with the commercial kits. The amount of RNA present was determined by real-time PCR TaqMan® assays using the blood- or semen-specific HBB or PRM2 probe/primers, respectively.

Based on preliminary experiments, the TRIzol® method was identified as an efficient and straight-forward procedure for the isolation of DNA and RNA. Despite the success of the TRIzol® extractions, there are several disadvantages which led us to actively seek an alternative isolation procedure. Although the TRIzol® reagent has the capacity to isolate RNA and DNA, this requires essentially two extraction pathways following the first initial steps. Therefore, we sought procedures which would be less labor-intensive and yield sufficient amounts of high-quality nucleic acids.

Various commercially available RNA/DNA isolation kits were tested to assess their capacity to isolate RNA (and DNA) in terms of yield and amplifiability. The methods tested were: Absolutely RNA® Miniprep Kit, AllPrep DNA/RNA Mini Kit, AquaRNA Kit, High Pure RNA Isolation Kit and the Sucrose Method. The experimental procedures were compared with our gold standard RNA and DNA extraction methods: TRIzol® Reagent and the validated DNA extraction method used by the Vermont Forensic Laboratory for casework samples ("Organic"). Although none of the new techniques surpassed the TRIzol® yields as determined by amplification of TaqMan® gene expression assays, the AllPrep and Absolutely RNA® kits were comparable. On the other hand, both the AquaRNA and HighPure kits were inferior in comparison. Additionally, the Absolutely RNA® kit was shown to be an efficient extraction method for human tissues, without the requirement of a homogenization step.

The AquaRNA and AllPrep kits are recommended for extraction of DNA as well, so they were compared to TRIzol® and the Organic method. The AquaRNA kit was not useful for DNA extraction. The AllPrep kit was comparable with the TRIzol® method although the yields were

significantly less than that of the Organic method. Therefore, the AllPrep kit was deemed a suitable alternative in place of the TRIzol® reagent for extraction of both DNA and RNA.

As the search went on for an extraction method that would work as well as TRIzol® for RNA and the Organic method for DNA, we came across a paper which outlined a simultaneous extraction method for both nucleic acids. Berendzen et al. (2005) developed a method utilizing a sucrose buffer to yield sufficient quality and quantity of RNA and DNA from plants. Unfortunately, the Sucrose Method wasn't an efficient RNA extraction method, however we were surprised at how well it performed DNA isolation. As a side investigation, the Sucrose Method performed as well or better than the Organic method or the BioRobot® M48 which we were using for CODIS samples at the time. There were no significant differences between the DNA yields from the three methods and importantly, the Sucrose Method yielded DNA of sufficient quantity and quality to perform downstream STR analysis (AmpFℓSTR® Identifiler™ PCR Amplification Kits). The Vermont Forensic Laboratory now processes all of its CODIS samples in-house using the Sucrose Method of DNA extraction with a 90-95% success rate (from extraction to upload).

We performed exhaustive studies to determine whether RNA extraction via the Sucrose Method was possible since the DNA extraction was shown to work so well. Changes were made to the length of the incubation with sucrose solution, the temperature during the incubations, RNase inhibitors were added to the solution and the sucrose solution was added pre-heated. RNA stabilization reagents and proteinase K were utilized, but none of these permutations had a significant effect on the DNA yields. However, through these experiments we were able to develop, optimize and validate a new DNA extraction technique which has led to significant savings in terms of analyst time and reagent costs. Also, we were able to rule out a number of

commercial kits that were recommended extraction methods. Future studies in this grant utilized the TRIzol® method and Absolutely RNA® kit for RNA extraction, as well as the AllPrep Kit for dual extraction of RNA and DNA.

The second goal of the project was to determine the half-life of mRNA in different types of stains under different conditions. Prior to and throughout the course of the grant we have been collecting samples (blood, seminal fluid, saliva, urine) in order to determine the stability of candidate mRNAs for stain identification. In order for a candidate to be implemented into a stain identification assay, it needs to be detectable for years following deposition of the sample. It was long believed that RNA was very unstable and difficult to work with, requiring dedicated equipment and laboratory space, however recent work has shed doubt on that theory. To determine the amount of amplifiable RNA, we performed real-time PCR using TaqMan® Assays-on-Demand™ Gene Expression Products.

In support of the view of RNA as a stable entity, we used amplification of HBB and PRM2 genes as an indicator of mRNA stability in blood and semen samples, respectively. Regardless of the sample size (i.e. down to 1 µl), we were able to detect these genes in samples aged well over 3 years. Following amplification with the TaqMan® HBB assay, it was apparent that this candidate gene was very stable over time. Likewise, PRM2 was amplified from RNA extracted from the first sample collected for this project, a seminal fluid stain aged for 1666 days at room temperature in the dark, with little difference in Cts from those of fresh samples.

Since forensic samples are often exposed to a variety of environmental insults, we stored parallel blood and semen samples under ideal conditions (room temperature, dark) as well as in a sub-

optimal condition (37˚C). By comparing gene amplification between the samples, we hoped to determine whether RNA profiling would be feasible for weathered forensic stains. There was no considerable decrease in the degree of amplification after prolonged exposure (>500 days) to elevated temperatures. Regardless of the storage length or conditions, the resulting Cts were essentially the same with a difference of less than 1 Ct between all fresh and aged samples. These results further demonstrate how stable HBB and PRM2 RNA are over time. In conclusion, our results indicate that RNA can be recovered from forensically relevant biological stains in sufficient quantity and quality for mRNA analysis.

The third goal of this project was to identify 2-3 gene candidates which were specific for each tissue of interest. The candidates used throughout the course of the grant were identified through surveys of literature (PubMed), Gene and other databases. Initially we identified 2-3 genes that appeared to be specific for each tissue. These screening studies were performed using TaqMan® primer/probe sets from Applied Biosystems because they were pre-designed, inexpensive and thoroughly tested for specificity. We tested the specificity, robustness and sensitivity of each assay by analysis of mRNA isolated from the body fluid of interest. Once the assay was shown to be robust, we tested it on mRNA isolated from other fluids and tissues to demonstrate that the assay is specific. Our diverse sample bank was used to assess the specificity of the candidate tissue-specific genes. These samples included blood, semen, saliva, menstrual blood, vaginal secretions, kidney, colon, adipose, skin, and control human RNAs (brain, heart, liver, kidney, intestine).

A sensitivity study of the three candidate blood assays (HBB, CD3G, SPTB) demonstrated how minute volumes of blood could be detected using mRNA profiling. Amplification with the HBB

probe/primers occurred with as little as 0.0001 µl of blood. Although the seminal fluid genes were not as sensitive as the HBB assay, amplification using the PSA and PRM2 TaqMan® sets occurred with 0.01 µl of semen. In an attempt to identify the lower detection limits of our seminal fluid assays, known quantities of a control testes sample were used. Both PRM2 and TGM4 were detected in 1ng of RNA, whereas MSMB and SEMG1 were detectable at 10 ng and 100 ng, respectively. Although our goal of developing screening assays does not involve quantitation of our samples, this experiment demonstrated that TGM4 and PRM2 are robust candidates for the detection of semen or sperm, respectively.

The sensitivities for saliva candidate markers (STAT, HTN3) were not as low as found for the blood and semen assays; 1 µl for STAT and 5 µl for HTN3. However, the result is significant because there are no Cts observed for the negative controls. The two urine TaqMan® assays (REN and UPK2) were unsuccessful using 20 µl urine stains. Therefore, other potential candidates need to be identified for urine.

The specificity of the chosen genes for the tissue of interest was also examined. To test the TaqMan® sets (only the control B2M and tissue-specific sets should give amplification), mRNA was isolated from dried blood, semen, saliva, menstrual blood or vaginal secretions using TRIzol®. Human tissues (kidney, colon, adipose, skin) were extracted using the Absolutely RNA® Kit. Control human RNAs from Ambion (brain, heart, liver, kidney, intestine) were diluted to 100 ng/µl. The RNA samples were then reverse transcribed and PCR performed with each of the TaqMan® sets.

The fluid specificity results were fairly straight-forward. HBB, SPTB and CD3G (blood markers) only amplified in blood samples, whereas STAT (saliva marker) only amplified in saliva samples. There was some cross-reactivity with the semen candidates. Some minor amplification occurred with vaginal secretions (SEMG1 and ACPP) and blood (ACPP). But, for the most part, the assays only amplified from semen stains. Based on these results, several of these TaqMan® assays appear to be specific (HBB, SPTB, CD3G, PSA, STAT), however, screening against an expanded sample set would strengthen that claim. Alternatively, TGM4 and PRM2 have demonstrated their specificity for semen/sperm through the course of these studies.

On the other hand, the specificity studies using the tissue assays yielded mixed results. For all but one of the tissues (heart), there was cross-reactivity with nearly all of the candidates tested. The most challenging tissues to identify specific markers for were kidney and liver. Furthermore, even when some candidates appeared to be specific based on the results using the control RNAs, differing results were seen with actual human tissue samples. Such was the case with intestine and kidney genes. All 3 intestinal markers amplified solely from the control intestine sample, yet amplification occurred with human colon and kidney tissues. Similarly, CLCNKA amplified with only the control kidney RNA, yet was picked up in each of the human tissues tested. Therefore, before any claims can be made regarding the specificity of the tissues candidates, experiments using actual human tissues (like what was done for kidney and colon/intestine) need to be performed. We have not been able to obtain human heart, brain or liver samples through our collaborators to date, but we are optimistic they will be obtained in the near future.

A major aim of stain identification using mRNA expression profiling, and the fourth goal of the project, is working towards multiplexing the real-time PCR assays once mRNAs are identified that clearly define specific types of stains. The Plexor® system from Promega was one technology we employed to develop multiplex assays. Depending on the dye-capability of the real-time instrument that is utilized, this system allows multiple mRNAs to be combined in one assay, thus reducing the amount of sample needed and time of analysis. For example, using a 4-dye channel instrument, eight mRNA targets could be analyzed; two per dye-channel, each of which has a distinctive melt curve.

Our initial focus was to design a blood-semen stand alone assay composed of two blood-specific (HBB, CD3G) and two semen-specific (PSA, PRM2) genes. While the HBB primer sets worked well to amplify from blood samples, we ran into primer-dimer and genomic DNA amplification problems with the PSA primers which were overcome by changing primer concentrations and cycling conditions. The PRM2 Plexor® primers worked extremely well to amplify from semen samples with virtually no amplification from the non-semen samples or negative controls. We were unable to optimize conditions for the other blood marker, CD3G, so we didn't pursue it as part of the blood-semen assay. As a 3-plex assay, we were able to show that these primer sets were able to discriminate their target RNA in a heterogeneous sample of blood and semen RNA extracts. Although this assay has yet to be tested and optimized for mock casework samples, we feel that it has potential as a screening tool for blood and semen identification.

A second multiplex assay which we designed based on the Plexor® technology was a seminal fluid assay, since seminal fluid analysis is a major task for all forensic laboratories. For semen, we were interested in genes specific for sperm and prostatic components. An assay that could

determine whether the stain 1) is seminal fluid and 2) contains sperm could alleviate the need to perform extensive microscopy for the identification of sperm. During the course of optimizing the seminal fluid assay, we settled on a triplex consisting of ACPP, PRM2 and GAPDHS; one semen- and two sperm-specific markers. All three primer pairs amplified from semen and a 1:1 mixture of blood and semen to a greater extent than blood alone or the negative controls. In order to 4-plex, we labeled PSA primers with Quasar 670 (CY5). In order to overcome the amplification of genomic DNA observed previously with these primers, we DNase treated our samples. However, the addition of a DNase treatment step is one to be avoided since it adds a 30 minute step, often results in a loss of RNA, and should not be used in close proximity to evidentiary DNA samples. The multiplex consisting of ACPP, PSA, GAPDHS and PRM2 primer sets is promising. Further optimization of the assay is needed, but all four primer sets appear to work in the presence of each other to discriminate between semen and non-semen samples.

The third Plexor®-based multiplex assay arose out of collaboration with Promega to develop stain identification assays. The goal of the collaboration is to generate a panel of Plexor® Tissue Typing Systems; multiplexed qRT-PCR systems for determining the source and quantity of a variety of human stains and/or tissues. By limiting the system to two-color detection (i.e. FAM and HEX detection), it will be compatible with the majority of real-time thermal cyclers currently in place in forensic laboratories. We sought to include controls (e.g. a housekeeping gene) and multiple, tissue-specific targets using the ability of the Plexor® Data Analysis Software to distinguish two different amplicons in the same dye channel, based upon thermal melt properties.

The Stain ID assay currently in the final stages of development detects semen and saliva using the targets TGM4 (semen), HTN3 (saliva) and GAPDH (housekeeper). Through numerous optimization studies, we generated saliva primers that show specific reactivity with saliva RNA and no cross-reactivity with other RNA samples or with genomic DNA. The semen primers (TGM4) show specific reactivity with semen RNA and no cross-reactivity with other RNA samples or with genomic DNA. Titration experiments indicate that semen has a detection threshold of approximately 10 pg of RNA whereas the threshold for saliva is approximately 1 ng of RNA. Furthermore, we assessed how the Stain ID assay would perform with typical forensic samples (i.e. various volumes of unknown RNA yields). The only non-specific amplification that occurred was from the HTN3 primers and the vaginal secretions sample. Since this experiment was performed, the HTN primers have been modified and no longer detect vaginal secretions.

A major outcome of the Stain ID assay development was the generation of a co-isolation method for RNA and DNA extraction; one of the goals of this project. By utilizing one extraction step, a DNA sample would be ready and waiting for STR profiling if the RNA screening assay deemed it worthy of such analysis. In addition, obtaining RNA and DNA from a single stain would prevent the possibility of conclusions being drawn regarding the identity of one stain which may not hold true for a nearby stain. Therefore, a significant amount of time was spent optimizing a procedure which would co-extract the two nucleic acids so that they were of sufficient quality and quantity for downstream analysis. A combination of the RNagents Total RNA Isolation System (Promega) and a Tris-buffered phenol protocol was optimized for simultaneous extraction of the nucleic acids. The final extraction method was shown to extract sufficient quantities of quality RNA and DNA from 1 and 10 µl of semen and saliva demonstrating its

utility as a dual extraction technique. Although this method involves numerous hands-on steps, it is faster than the TRIzol® method, and produces significantly better DNA yields. To date, it's the best co-isolation method we've tested in terms of yields and amplifiability. We are hopeful that the successful completion of these validation studies will result in the development of the first commercial RNA-based Stain ID kit for the detection of semen and saliva in forensic samples.

A major disadvantage to using the Gene Expression TaqMan® assays from Applied Biosystems is the inability to multiplex the assays since they are all labeled with the same dye. Therefore, we set out to design our own TaqMan® probes and primers in hopes of developing several specific multiplex assays. The first multiplex we designed was a semen-sperm detection assay using the TGM4 and PRM2 markers in addition to the housekeeper B2M. While each primer/probe set amplified from semen when alone in the reaction, the combination into a single multiplex reaction caused the dropout of TGM4 and B2M amplification. We are in the process of further optimization of this assay which is focusing on changing the primer and probe conditions since PRM2 is expressed at a much greater extent than TGM4 or B2M. Furthermore, changing the master mix used in this reaction may have an impact on the results, which has been observed in other studies in our lab.

The second TaqMan®-based multiplex assay we have developed is for the identification of brain tissue using ADCY1, GPM6A and the housekeeper B2M. Each primer/probe set amplified from brain both when alone in the reaction and in combination with the other primer/probe sets with no significant decrease in the degree of amplification. Further optimization is required to eliminate some minor amplification in the no template controls. Similar to the semen-sperm

assay, this multiplex looks very promising for a brain screening assay. Importantly, we hope to obtain human brain samples from collaborators which would assist in our evaluation of this multiplex as a viable screening assay.

A relatively new technology that may offer great promise towards multiplexing for the forensic community is the Bio-Plex™ system which uses the multiplexing technology of Luminex Corp. to enable the simultaneous quantitation of up to 100 analytes. The QuantiGene® Plex Reagent System is one platform which can be coupled with the Luminex instrument to enable simultaneous detection of multiple RNA targets directly from purified RNA. In order to determine whether this technology would work with routine forensic samples, we ordered a 3-plex assay to detect PRM2, SEMG1 and B2M. The most important finding from these preliminary studies was that samples prepared using a quick one-step lysis method fared better than samples where purified RNA was extracted using the TRIzol® reagent. As this multiplex assay was intended as a tool with which to evaluate the feasibility of this technology as a routine screening assay, we plan to design future QuantiGene® Plex assays based on the needs of the community. A disadvantage of this platform is the number of steps it requires; not a significant amount of hands-on time, but numerous incubations and wash steps performed using a vacuum manifold. However, since the capacity of this system is much more than other real-time RNA-based multiplex assays, there is a potential to generate large screening assays not possible with other techniques. Therefore, the QuantiGene® Plex assays should not be used for simple multiplex assays, but for large assays not compatible with conventional thermal cyclers, real-time instruments or capillary electrophoresis systems.

The final goal of this project was to disseminate our results to the forensic community. To this end we have published a paper outlining our work to identify specific gene targets for the purpose of multiplexing, presented our work at a number of scientific conferences, and intend on promoting a Stain ID assay which was developed out of collaboration with Promega.

MAIN BODY

I. Introduction

1. Statement of the Problem

In an age of scientific advances in molecular biology, DNA profiling has proven itself an invaluable tool in solving crimes. However, the potential exists for the tissue origin of the suspect DNA to be called into question. For example, in a court of law, a semen stain containing suspect DNA can have far more serious consequences than a saliva stain. Furthermore, it is paramount to the criminal justice system that suspect stains be identified definitively and accurately. Traditional serological approaches for stain identification often involve a presumptive color test, followed by a confirmatory test that typically employs a specific antibody designed to complex with a known protein; such as hemoglobin for blood and P30 or prostatic antigen for seminal fluid. In the past decade, unparalleled progress has occurred in the design of tests necessary to identify and individualize crime scene samples. While these testing methods have improved in simplicity and selectivity over the years, several problems still exist such as the possibility of cross-reactivity with other species and the lack of specificity for particular tissues. For example, the traditional test for saliva involves detecting the presence of the enzyme

amylase. While this enzyme is found in relatively high concentrations in saliva, it is also present at lower levels in other fluids. Therefore, definitively determining the presence of saliva is not possible by this method.

In order to develop more robust assays, we explored the possibility of using mRNA as a determinant of tissue specificity. Each tissue or cell type makes a unique constellation of mRNAs, some specific for only that tissue or cell type. An assay to detect these specific mRNAs will be indicative of the tissue of origin. Some body fluids, such as blood, contain cells as part of their function. Other fluids, such as urine, contain cells that have been shed from their tissue of origin. Thus, there is a strong possibility that a stain can be identified by determining which mRNAs it contains. Real-time reverse-transcription PCR is an extremely sensitive technique for the detection and quantitation of the tissue specific mRNAs. Assays using a real-time format could yield a quick and accurate identification of an unknown tissue or stain. The longer-term goal of this research was to multiplex several tissue assays to determine if the sample contains a mixture of tissues/stains or is from a single origin. The immediate goals of this research were to demonstrate that it is possible to isolate mRNA from a variety of body fluids, determine the specificity of certain mRNAs to each fluid and to examine the stability of these mRNAs over the course of time. The specific aims of this grant were 1) to select the best commercial kit to extract mRNA from a wide variety of stain types, 2) to determine the half-life of mRNA in different types of stains under different conditions, 3) to find 2-3 genes specific for each of four stain types (semen, blood, urine, saliva), 4) to develop multiplex assays for the identification of stain types, 5) to validate these assays for casework samples, and 6) to disseminate these results to the forensic community.

2. Literature Citations and Review

Although the DNA of all tissues from an individual is essentially identical, the RNA spectrum made by the different cells in each tissue is very different. Therefore, analysis of the RNA in a sample, especially identification of certain "tissue specific" mRNA transcripts, can provide an "RNA profile" which will uniquely identify the tissue of origin. A major interest in cancer research today is to determine the spectra of mRNAs made in different cell types in hopes of elucidating which changes turn normal cells into cancerous cells. For these studies, it is crucial to be able to distinguish the mRNA produced by normal cells compared to malignant cells. Thus, a body of knowledge is being developed regarding the distribution of various mRNAs in human tissues. Databases of information regarding the amounts of each mRNA present in every tissue have been and are being created. Examples include: NHGRI Tissue Microarray Project, Gene Expression Omnibus, BODYMAP and HugeIndex.

When molecular biologists began isolating mRNA for experiments, it was thought that mRNA was very ephemeral and that tissues needed to be processed rapidly in separate rooms with dedicated instruments and often with hazardous chemicals. However, due to the development of new techniques and the recent increase in knowledge concerning mRNA, it has been shown to be relatively stable. mRNA can be isolated from such items as formalin-preserved tissues and Guthrie blood spots (Liu et al., 2002; Fritsch et al., 2002; Macabeo-Ong et al., 2002; Matsubara et al., 1992; Cao and Cousins, 2000; Krafft et al., 1997; Lewis et al., 2001; Tetali et al., 2001; Pai et al., 1994; Cassol et al., 1997; Abe et al., 1998; Spielberg et al., 2000; Katz et al., 2002). For example, Tetali et al. typed individuals for the CCR5 32bp deletion using RT-PCR of blood spots that had been dried up to 12 months. Others have isolated mRNA from decade old samples

(Mizuno et al., 1998; Li et al., 1997) and mRNA has also been isolated from dried blood smears (Schoch et al., 2001; Crisan and Anstett, 1995).

A major obstacle which needs to be overcome in order for laboratories to invest in RNA technology is the development of a straight-forward extraction procedure. A method that could co-purify RNA and DNA from a single sample with a minimal number of steps would be attractive to those seeking new technologies. A number of methods describing the simultaneous isolation of DNA and RNA have been reported (Alvarez et al., 2004; Bauer and Patzelt, 2003; Chomczynski, 1993). However, most of these have not been optimized to deal with the reduced quantity or quality of samples encountered in forensic casework. Alternatively, the co-isolation reports using forensically relevant samples (Alvarez et al., 2004; Bauer and Patzelt, 2003) require numerous time-consuming steps that would not benefit fast and simple stain identification assays.

Many companies now sell kits for quick and easy isolation of DNA or RNA from a variety of sample types. However, despite the availability of simple and convenient commercial kits, little has been done in the forensic field to combine DNA and RNA extraction into a convenient dual extraction; specifically, to detect bodily fluid or tissue-specific mRNAs in crime scene samples. The standard commercial method of DNA/RNA co-extraction utilizes the TRIzol® Reagent from Invitrogen. Despite the quality of nucleic acids isolated using this method, a major disadvantage is the number or steps required making the process very time-consuming. A promising commercial extraction procedure is the AllPrep DNA/RNA Mini Kit from Qiagen. This kit is designed for purifying both DNA and RNA from a single sample in as little as 30 minutes with no need to divide the original sample into two for separate purification procedures. Although the

potential for DNA/RNA extraction from animal cells and tissue homogenates using the AllPrep kit had been demonstrated, its application to forensically relevant samples is widely unknown.

Groups have isolated mRNAs from blood, semen and saliva for research and diagnostic purposes. For blood, groups have isolated mRNA from dried blood spots for RT-PCR and restriction or quantitation (Matsubara et al., 1992; Cao and Cousins, 2000; Zhang and McCabe, 1992; Watanapokasin et al., 2000). For semen, a number of groups have developed methods to detect hepatitis C viral mRNA or HIV mRNA in seminal fluid (Bourlet et al., 2002; Dulioust et al., 1998). Researchers have also isolated nuclear mRNAs such as calcium channel subunit mRNAs from the sperm in semen (Goodwin et al, 2000). In fact, mRNAs of a number of genes have been found in human spermatozoa (Miller, 2000; Richter et al., 1999). In addition, beta-hCG mRNA has been isolated from the prostate cells in human ejaculate (Daja et al., 2000). For saliva, mRNA of viruses such as measles has been detected (Jin et al., 2002). In terms of forensic analysis, Bauer et al. (1999) have detected mRNAs specific for epithelial (endometrial) cells in menstrual blood samples. They found that they could isolate mRNA after 6 months of room temperature storage and detect a number of mRNAs species. Further study (Bauer and Patzelt, 2002) found that matrix metalloproteinase was a good marker for menstrual blood. Bauer et al. (2003) have a more recent paper where they studied 106 bloodstains stored up to 15 years. They found that mRNA levels as measured by laser-induced fluorescence capillary electrophoresis correlates with the age of the sample and that "mRNA suitable for RT-PCR can be isolated from samples stored up to 15 years".

Juusola and Ballantyne (2003) have isolated mRNA from blood, semen and saliva stains and used it for RT-PCR of the control genes S15, beta-actin and GAPDH. In addition, they studied

the saliva specific genes statherin, histatin 3, PRB1, PRB2 and PRB3. mRNA for these latter genes was found only in the saliva stains.

Technology in the field of multiplexing gene expression assays is rapidly improving. The Plexor® One-Step qRT-PCR System takes advantage of the specific interaction between two modified nucleotides to achieve quantitative PCR analysis. It is possible to design assays to quantify multiple targets within the same reaction using primer pairs with a different fluorophore for each target sequence. Therefore, you are limited by the dye capability of your real-time PCR instrument. However, since DNA quantitation is moving towards a molecular biological approach which utilizes three- or four-dye real-time PCR instruments, most labs could implement this technology without purchasing new equipment.

A new technology that may offer great promise to the forensic community is the Bio-Plex™ system which uses the multiplexing technology of Luminex Corp. to enable the simultaneous quantitation of up to 100 analytes. This technology uses polystyrene beads (xMAP® beads) internally dyed with differing ratios of two spectrally distinct fluorophores. Each fluorophore can have any of 10 possible levels of fluorescent intensity, thereby creating a family of 100 spectrally addressed bead sets. Multiplex assays can be created by mixing bead sets to simultaneously test for many analytes in one sample. This valuable technique could be used in routine testing and could assess many samples in an automated fashion.

Another adaptation to the Luminex-bead technology is the QuantiGene® Plex Reagent System offered by Panomics. This system combines branched DNA with the multi-analyte profiling beads. Together they enable simultaneous detection of multiple RNA targets directly from

purified RNA. Branched DNA technology is a sandwich nucleic acid hybridization assay that amplifies the reporter signal rather than the sequence. Groups have been able to utilize this technology to measure gene expression from blood (Zheng et al., 2006), formalin-fixed, paraffin-embedded tissues (Yang et al., 2006), and directly from cell lysates and tissue homogenates without the need for RNA purification (Zhang et al., 2005; Flagella et al., 2006).

Altogether these studies and commercially available techniques indicate that DNA and RNA can be co-extracted and the RNA fraction used in multiplexed real-time PCR assays. We proposed to develop real-time PCR assays to detect tissue-specific transcripts for human fluids and tissues. These assays would ultimately be multiplexed for faster determination of tissue origin. A major advantage to these assays is that a single sample extract will be used to classify the sample as blood, semen, vaginal secretions, brain, heart, etc. which will drastically reduce the number of identification tests performed prior to DNA profiling (if required).

3. Rationale for the Research

The implementation of DNA analysis within the forensic laboratory has been a tremendous benefit to the criminal justice community. However, as technologies progress, there are more cases and items per case requiring DNA analysis and, unfortunately, the manpower and resources needed to work the cases have not kept up with demand. The research into stain identification using real-time PCR seeks to find faster and more efficient methods to work DNA cases. The goal is to develop methods that broaden real-time PCR applications and to investigate a new technology that could radically change the analysis of biological stains and tissues. The biochemical approaches currently used in tissue identification are limited in scope and often

imply, but not truly identify, the source fluid/tissue. Many believe that the positive identification of fluids and tissues can be performed quickly and efficiently, and that tissues not routinely evaluated could be easily assessed by all laboratories so that an equality of testing could be realized across the country. The evaluation of mRNA through real-time PCR will be a technique that can offer a level of confidence and expand our knowledge of the materials we routinely examine. This research into new technologies will demonstrate the power of multiplexing for forensic analysis.

As the demand for sample analysis increases, forensic laboratories continue to balance manpower and cost issues versus the value of the analysis when evaluating new techniques. In order for laboratories to invest in RNA technology, they will require a straight-forward extraction procedure. Furthermore, a crucial prerequisite to these analyses is the development of a DNA/RNA co-extraction method to minimize sample requirements and eliminate the need for two separate extractions. A method that could co-purify RNA and DNA from a single sample with a minimal number of steps would be attractive to those seeking new technologies. A number of methods describing the simultaneous isolation of DNA and RNA have been reported, however, most of these have not been optimized to deal with the reduced quality of samples encountered in forensic casework. Alternatively, the co-isolation reports using forensically relevant samples require numerous time-consuming steps that would not benefit a fast and simple stain identification assay. A preferred extraction method would demonstrate the best ability to co-isolate DNA and RNA in terms of yield and amplifiability while remaining simple, efficient, and ideally, involve non-hazardous reagents.

The purpose of this RNA-based stain identification research is to develop a simple mRNA extraction and analysis method to allow the quick, unequivocal identification of body fluid stains and tissues. The proposed assays will be designed so that a single stain will amplify with only the corresponding mRNA(s) of its fluid/tissue type; a mixture, however, should amplify representing various fluids/tissues. The assays are designed to function more qualitatively than quantitatively, so although they may indicate which fluids/tissues are present, they may not give the exact ratio of each fluid present. However, it is of greater concern to know what kind of mixture exists, than to know the exact amount of each fluid present. Furthermore, a major advantage to these assays is that a single test will be used to classify the sample as blood, semen, vaginal secretions, brain, heart, etc. which will drastically reduce the number of identification analyses performed prior to DNA profiling (if required).

II. Methods

Sample Preparation

Blood, seminal fluid, saliva and urine samples were collected over the course of the grant by pipetting known amounts of fresh fluid onto swatches of cotton cloth or various other surfaces (denim, cardboard, vinyl, metal, carpet) and allowing the spots to dry at room temperature. Vaginal swabs were collected on cotton swabs, whereas menstrual blood was collected on tampons and allowed to dry at room temperature. Each sample was stored in a glassine envelope, which was kept at room temperature in a box designated for the particular fluid at the Vermont Forensic Laboratory. Some samples were also aged at 37°C in the dark. Human

tissues (kidney, colon, adipose, skin) were collected from the Autopsy Service and Surgical Pathology Suite at Fletcher Allen Health Care and frozen at -20˚C until use.

Human control RNAs (brain, heart, liver, kidney, intestine) were purchased from Ambion (Austin, TX) for use in several experiments. These RNAs were supplied as 1 µg/µl and diluted as noted for experiments.

RNA/DNA Extraction

Various commercially available RNA/DNA isolation kits, as well as several homebrew methods were tested to determine which gave the best RNA/DNA yields for the fluids and tissues under consideration. The methods tested were:

Absolutely RNA® Miniprep Kit (Stratagene, La Jolla, CA) This method employs a spin cup with a silica-based fiber matrix that binds RNA in the presence of chaotropic salt while a series of washes removes contaminants. The lysis buffer contains guanidine thiocyanate to lyse the cells and to prevent RNA degradation by RNases. Following cell lysis, the sample is prefiltered in a spin cup to remove particles and to reduce the amount of DNA. The filtrate is then transferred to a spin cup with a silica-based fiber matrix which binds the RNA. Treatment with a low-salt wash buffer and digestion with DNase removes the remaining DNA. A series of washes removes the DNase and other proteins. Highly pure RNA is eluted from the fiber matrix with a small volume of RNase-free water and captured in the microcentrifuge tube.

AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA) Biological samples are lysed in a guanidine-isothiocyanate buffer, which immediately inactivates DNases and RNases to ensure isolation of intact DNA and RNA. The lysate is then passed through an AllPrep DNA spin column. This column, in combination with the high-salt buffer, allows selective and efficient binding of genomic DNA. The column is washed and pure, ready-to-use DNA is then eluted. Ethanol is added to the flow-through of the AllPrep DNA spin column to provide appropriate binding conditions for RNA, and the sample is then applied to an RNeasy spin column, where total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in RNase-free water.

AquaRNA Kit (MultiTarget Pharmaceuticals, Salt Lake City, UT) This kit is composed of a multifunctional aqueous solution containing guanidine isothiocyanate. The AquaRNA solution lyses the cells, inactivates the RNases, and extracts DNA, RNA and proteins in one step. Total DNA/RNA is precipitated from the lysate with isopropanol. Several washes with ethanol follow with a final step to reconstitute the pellet with water.

High Pure RNA Isolation Kit (Roche, Mannheim, Germany) Samples are lysed by a special buffer containing guanidine hydrochloride which also inactivates RNases. Nucleic acids are bound to the glass fibers pre-packed in a filter tube. Residual contaminating DNA is digested by DNase I, applied directly to the glass fiber. Bound nucleic acids are washed to eliminate inhibitory contaminants. Subsequent washing of the bound nucleic acids purifies the sample of salts, proteins and other cellular impurities. The RNA is recovered using an elution buffer.

Organic Method (Vermont Forensic Laboratory, Waterbury, VT) Samples are lysed in stain extraction buffer containing detergents and proteinase K overnight at 56°C. Phenol:chloroform:isoamyl alcohol is added to the lysed samples and following centrifugation, the DNA aqueous phase is separated from the organic solvent. The samples are purified using Microcon 100 Concentrators (Millipore Corporation, Bedford, MA) and the DNA is eluted in TE^{-4}.

PureYield™ RNA Midiprep System (Promega, Madison, WI) This system is designed to quickly and easily isolate high yields of pure total RNA while eliminating the co-purification of DNA. The protocol uses the PureYield™ silica-membrane technology to isolate intact RNA. The PureYield™ RNA Midiprep System provides many unique features to purify total RNA without using DNase treatment, phenol:chloroform extractions, protease digestion or alcohol precipitations. The PureYield™ RNA Midiprep System avoids the problems routinely involved with DNA contamination and its subsequent removal by selectively eliminating DNA prior to total RNA isolation, using the PureYield™ Clearing Agent, which preferentially binds DNA leaving the RNA virtually free of DNA.

QuantiGene 2.0 Direct Lysis Sample Preparation (Panomics Inc., Fremont, CA) Samples are transferred to microcentrifuge tubes and combined with a Working Lysis Mixture containing proteinase K and other proprietary components. The samples are incubated at 60°C for 30 minutes and the lysates are removed to a clean tube for use in QuantiGene assays.

RNAgents® Total RNA Isolation System (Promega) This procedure utilizes the RNAgents® Denaturing Solution to lyse cells or tissue under conditions that rapidly inhibit ribonucleases

using two potent inhibitors of RNase, quanidine thiocyanate and β-mercaptoethanol. The Solution is designed to be used in concert with acidic phenol:chloroform and alcohol (isopropanol) for purification of total RNA.

Sucrose Method (Vermont Forensic Laboratory) Samples are incubated with Sucrose Solution (sucrose, Tris-HCl and NaCl) for 10 minutes at 100˚C and purified using a MultiScreen filter plate (Fisher, Pittsburgh, PA) and vacuum filtration.

Tris-Buffered Phenol Method (Stain ID extraction) (Promega and Vermont Forensic Laboratory) This protocol is optimized for the simultaneous extraction of both DNA and RNA from a sample. The resulting total nucleic acid is suitable for analysis using both the Stain ID system as well as Promega's STR systems. The procedure uses a guanidine thiocyanate-based denaturing solution in combination with a Proteinase K treatment step to provide rapid sample lysis and protein precipitation, effectively protecting the RNA during sample lysis. A Tris-buffered phenol extraction step efficiently extracts both DNA and RNA from the lysed sample. Alcohol precipitation serves to wash the total nucleic acids of residual salts.

TRIzol® Reagent (Invitrogen, Carlsbad, CA) This reagent is a monophasic solution of phenol and guanidine isothiocyanate suitable for isolating total RNA, DNA, and proteins. During sample lysis, TRIzol® Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl

alcohol. After removal of the aqueous phase, the DNA in the sample can be recovered by precipitation with ethanol to yield DNA from the interphase.

Reverse Transcription

Reverse transcription was performed on isolated RNA using the MessageSensor™ RT Kit (Ambion). This kit enables the synthesis of cDNA from total RNA, poly(A) RNA, or from a purified RNA transcript for use in PCR. It employs MMLV reverse transcriptase that maintains full RNase H activity for maximum sensitivity. cDNA created by this procedure was used in real-time PCR as described below.

TaqMan®-Based Real-Time PCR

Singleplex assays using real-time PCR on cDNA were performed using Assays-on-Demand™ Gene Expression Products (Applied Biosystems, Foster City, CA) (Table 1a). These are a comprehensive collection of pre-designed and tested primer and probe sets that allow researchers to perform quantitative gene expression studies on any human gene. They are designed against GenBank transcripts, transcripts from the Mammalian Gene Collection, and human Celera transcripts. Each assay is built on 5' nuclease chemistry and consists of two unlabeled PCR primers and a FAM™ dye-labeled TaqMan® minor groove binder (MGB) probe. The components are formulated into a single 20X mix and designed to run under universal conditions for reverse transcription and PCR. Assays with "m1" in the ID code indicate an assay whose probe spans an exon junction and therefore is designed to amplify only target cDNA without amplifying genomic DNA. This is the result of targeting primer sites that span regions of mRNA

in which introns have been removed, making the mRNA different from the DNA that it originated from. Those with "g1" in the ID code may possibly amplify genomic DNA.

The TaqMan® probe consists of two types of fluorophores, which are the fluorescent parts of reporter proteins (Figure 1). While the probe is attached or unattached to the template DNA and before the polymerase acts, the quencher (Q) fluorophore reduces the fluorescence from the reporter (R) fluorophore. It does this by the use of fluorescence resonance energy transfer (FRET), which is the inhibition of one dye caused by another without emission of a proton. The reporter dye is found on the 5' end of the probe and the quencher at the 3' end. Once the TaqMan® probe has bound to its specific piece of the template DNA after denaturation and the reaction cools, the primers anneal to the DNA. Taq polymerase then adds nucleotides and removes the TaqMan® probe from the template DNA. This separates the quencher from the reporter, and allows the reporter to give off its energy which is quantified using a computer. The more times the denaturation and annealing takes place, the more opportunities there are for the TaqMan® probe to bind and, in turn, the more emitted light is detected.

In addition to the Assays-on-Demand™ gene expression products purchased from Applied Biosystems, we designed our own in-house TaqMan® assays for seminal fluid/sperm and brain. Primers and TaqMan® probes were designed using the Beacon Designer program (PREMIER Biosoft International, Palo Alto, CA). Primers were made for PRM2 (F, AGTCACCTGCCCAAGAAACAC; R, ACTTTTGCTCGTTTCACTCAGATC), TGM4 (F, CTGGATGAAGCGACCGGATC; R, ATGTCACCTTTGCGGATGGC), B2M-Semen (F, TCCTGAAGCTGACAGCATTCG; R, GGATGACGTGAGTAAACCTGAATC), ADCY1 (F, CCTGCTGTCAACCTCTCCTC; R, CTAGTGGAAAGGGGACCATAAGG), GPM6A (F,

CACCTCACTGCCAGTTTACATG; R, TCACAATTCCAAACTGACGAAGG) and B2M-brain (F, CATTCGGGCCGAGATGTCTC; R, TGCTGGATGACGTGAGTAAACC) and purchased from Biosearch Technologies (Novato, CA). TaqMan® probes (PRM2, CTTCTCGGCGGCAACTCAGGGCT; TGM4, CCCAAGGGCTACGACGGCTGGC; B2M-Semen, TGTCTCGCTCCGTGGCCTTAGCTG; ADCY1, CTGCCTTGTCCCTGCTCCTGTGCT; GPM6A, TGTGGACCATCTGCCGGAACACCA; B2M-Brain, TGGCCTTAGCTGTGCTCGCGCT) were labeled with FAM (PRM2, ADCY1), CAL Orange 560 (TGM4, GPM6A) or CAL Red 610 (B2M-Semen, B2M-Brain) on the 5'end and were also purchased from Biosearch Technologies.

Plexor®-Based Real-Time PCR

Eragen Biosciences developed and synthesized a series of new DNA base pairs (Figure 2). The Plexor® technology (Promega) is based on one of these new base pairings: isoG and isoC. IsoC and IsoG nucleotides are incorporated by DNA polymerase; however, neither isoC nor isoG can base pair with any of the other conventional bases. These two novel bases only interact and base pair with one another and are not found in nature. Although similar to the conventional G-C pair, you can see the hydrogen bonding pattern is much different.

The Plexor® assay uses two primers that are specific for the target of interest. One of the primers contains a 5' modified nucleotide (iso-dCTP) linked to a fluorescent label. The second primer is unlabeled. The fluorescently labeled C residue only pairs with iso-dG, not regular G residues. The Plexor® primer and the normal downstream primer begin the process of replicating the DNA sequence of interest into new double-stranded template (Figure 3). The process is fed by

conventional dNTPs, as with any amplification. At the end of the amplimer the polymerase is confronted with the isoC base. The Plexor® System master mix includes iso-dGTP bound to the quencher dabcyl. In subsequent rounds of PCR, iso-dG is incorporated into the new DNA strand opposite from iso-dC, bringing the quencher into close proximity with the fluorescent dye, resulting in very efficient quenching of the fluorescent reporter. The fluorescent signal decreases in direct proportion to the amount of PCR product made. The number of cycles required to reach a significant decrease in fluorescence, the cycle threshold (Ct), is dependent on the amount of template DNA present. The amount of template DNA can be quantitated by comparison to a standard curve generated from known amounts of target DNA. As a quality check, the Plexor® Systems allow you to measure the melting temperature of the PCR products. Homogeneous product creates a well defined melting curve. The Plexor® methodology lends itself to multiplex real-time amplification.

A single Plexor® reaction can contain multiple Plexor® primer sets; each primer pair is specific to a different target sequence, and labeled with different fluors. The dabcyl-iso-dGTP in the Plexor® master mix will quench the fluorescence of all the dyes present in the reaction. Plexor® primers for homebrew assays were designed using the Plexor® Primer Design Software (Promega). Primers were made for HBB (F, isoC-GGGCAAGGTGAACGTGGATGAA; R, CCAAAGGACTCAAAGAACCTCTGG), CD3G (F, isoC-CAGAAGTACCGAACCATCTTCTTGA; R, CCTGGCTATCATTCTTCTTCAAGGT), PSA (F, isoC-CCAGCCTCCCACAATCCGAGA; R, TGCACCCGGAGAGCTGGTGTCA), PSA-redesign (F, isoC-CTGAAATACCTGGCCTGTGTCTTC; R, CAGTCTGCGGCGGTGTTCTG), PRM2 (F, isoC-GCGACTTTTTCTTAATTTCCAGCTGG; R, GCAAAAGACGCTCCTGCAGG), ACPP (F, isoC-

CTTCTTGTTTCTGCTTTTTTTCTGGCTAG; R, GGTCAGTGGGAAAGGTGTCAATG), SEMG1 (F, isoC-GTACTTTCCCTGCTCCTCATCTTG; R, TTGTTGCTTGCCTTTTTGTCCAGA), GAPDHS (F, isoC-TGACTGGTGTGGGCTCTGGTT; R, CCAATGTCACCGTTGTCCAGT) and purchased from Biosearch Technologies. The primers containing the isoC were labeled with FAM (HBB, GAPDHS), CAL Orange 560 (PSA, PSA-redesign, ACPP), CAL Red 610 (CD3G, PRM2, SEMG1) or Quasar 670 (CD3G, PSA-redesign, PRM2) on the 5'end and were also purchased from Biosearch Technologies. The Plexor® primers for the Stain ID assay were designed by Promega using the Plexor® Primer Design Software (Promega). However, since the kit is in the development phase, we are unable to publish the primer sequences at this point in time.

Luminex Technology for Multiplexing

The xMAP® (multi-analyte profiling) system, developed by Luminex Corp, combines flow cytometry, fluorescent-dyed microspheres (beads), lasers and digital signal processing to effectively allow multiplexing of up to 100 unique assays within a single sample. The xMAP® technology uses 5.6 micron polystyrene microspheres which are internally dyed with red and infrared fluorophores. Using different intensities of the two dyes for different batches of microspheres, the creation of 100 xMAP® microsphere sets, each with a unique spectral signature determined by its red/infrared dye mixture was possible. The beads are filled with a specific known ratio of the two dyes. As each microsphere carries a unique signature, the xMAP® detection system can identify to which set it belongs. Additionally, each microsphere set contains a different anti-tag sequence which serves as functional groups to capture molecules

containing complementary tag sequences that are introduced onto a cDNA of a gene of interest. Therefore, multiplexing up to 100 tests in a single reaction volume is possible.

PCR is performed using two primers flanking each gene of interest. As shown in Figure 4, two probes are present in the second reaction: each one contains a distinct tag attached to a sequence complementary to the amplified gene of interest. In the allele-specific primer extension (ASPE), a DNA polymerase is used for primer extension and biotin-dCTP label incorporation. The tagged, biotin-labeled products are captured by their tag complements (anti-tags) on xMAP® microspheres. Streptavidin-phycoerythrin (PE) is used to indirectly detect the ASPE-incorporated biotin. The Bio-Plex™ 200 (i.e. BioRad's version of the Luminex 200; Figure 5) sorts the bead sets and provides a read-out of what bead set(s) (i.e. mRNAs) are present in the reaction sample.

As in any flow cytometer the stream of suspended particles (beads) is lined up in single file prior to passing through the detection chamber. This approach allows each particle to be measured as a discrete event. Each particle is simultaneously exposed to a red (bead classifier) and green (reporter quantifier) laser which decode both the signature of the individual bead and the code specific to the concentration of analyte associated with that bead. As a bead passes through the detection chamber, a red laser excites both dyes and decodes the bead for identification based on the ratio of the two dyes excited. A green laser excites the orange fluorescence associated with the analyte of interest, and quantifies the analytes.

QuantiGene® Plex 2.0 assays (Panomics Inc.) combine branched DNA (bDNA) signal amplification technology and xMAP® beads to enable simultaneous quantification of multiple

RNA targets directly from cultured cell or whole blood lysates; fresh, frozen or formalin-fixed, paraffin-embedded tissue homogenates, or purified RNA preparations. Branched DNA technology is a sandwich nucleic acid hybridization assay that provides a unique approach for RNA detection and quantification by amplifying the reporter signal rather than the sequence (Figure 6). By measuring the RNA at the sample source, the assay avoids variations or errors inherent to extraction and amplification of target sequences. The bioinformatics division at Panomics designs all of the assay components which has the advantage of saving the analyst valuable time, however, the downside is that all sequences remain proprietary to Panomics.

Specific mRNA transcripts are captured to their respective beads through a Capture Extender (CE) - Capture Probe (CP) interaction during an overnight hybridization at 53˚C. This is followed by sequential hybridization of the bDNA amplification molecule and biotinylated Label Probe, respectively, for an hour at 46˚C. Binding with Streptavidin-conjuagted Phycoerythrin (SA-PE) occurs at room temperature for 30 minutes. The sample is then analyzed on a Luminex instrument and the level of SA-PE fluorescence is proportional to the amount of mRNA transcripts captured by the bead.

DNA Quantitation

The TaqMan® duplex human/Y DNA quantitation assay was used to compare the DNA isolation methods. This technique was developed by Nicklas and Buel (2006) and is based on PCR amplification of the *Alu* sequence and the Y chromosome-specific DYZ5 sequence. A dilution series of human genomic DNA ranging from 64 ng/µl to 0.0039 ng/µl, along with a negative

control is run with each assay. The DYZ5 FAM-labeled probe fluorescence was read in the FAM channel while the VIC-labeled *Alu* probe fluorescence was read in the HEX channel.

Real-time PCR experiments were conducted in a RotorGene 3000 or 6000 (Corbett Research, Sydney, Australia) and data was analyzed using software RotorGene 4.6 from Corbett Research. The instrument uses the Ct values of the dilution series to generate a standard curve from which the concentrations of the unknown samples are automatically determined.

STR Analysis

DNA was amplified using AmpFℓSTR® Identifiler™ PCR Amplification Kits from Applied Biosystems in a GeneAmp PCR System 9700 (Applied Biosystems). The Identifiler™ kit amplifies 15 loci (*D8S1179, D21S11, D7S820, CSF1PO, D3S1358, THO1, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA*) and *amelogenin*. However, the *D2S1338* and D19S433 loci are not part of the core CODIS loci and therefore, are not factored into the quality control portion of STR interpretation. PCR reactions were performed as per manufacturer's protocol, using 1 ng of DNA in a 25 ul reaction volume under standard cycling conditions. One µl of each amplification product was analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems) as per manufacturer's protocol, using software 3130 Data Collection v3.0 (Applied Biosystems) and GeneMapper® ID v3.2 Software (Applied Biosystems) to analyze the data.

III. Results

1. Statement of Results

Aim #1: To select the best commercial method to extract mRNA from a wide variety of stain types.

Our first goal was to identify the best method to extract RNA from a variety of stain types. Through the course of the grant, we tried several commercial extraction kits which had various degrees of success. In addition, we sought a protocol which would allow the simultaneous or co-extraction of DNA. This is especially important for small stains. If RNA is isolated and the stain type is deemed worthy of STR analysis, it is crucial that there is enough sample left to obtain a profile or the analysis might prove to be a waste of time. Parallel stains were made with a measured amount of blood or semen and RNA isolated with the commercial kits. The amount of RNA present was determined by real-time PCR TaqMan® assays using the blood- or semen-specific HBB or PRM2 probe/primers, respectively.

Based on preliminary experiments, the TRIzol® method was identified as an efficient and straight-forward procedure for the isolation of DNA and RNA. RNA was isolated from freshly dried blood spots (0.1-20 µl) and used in RT reactions prior to performing PCR with the TaqMan® HBB assay. The results shown in Figure 7 demonstrate the success of the extraction based on the low Ct values.

Despite the success of the TRIzol® extractions, there are several disadvantages which led us to actively seek an alternative isolation procedure. The TRIzol® reagent has the capacity to isolate RNA and DNA; however, this requires essentially two extraction pathways following the first initial steps. In addition to being labor intensive, the procedure involves the use of hazardous chemicals which require special safety and handling concerns. Therefore, we sought procedures which would be less labor-intensive, involve non-hazardous reagents, and yield sufficient amounts of high-quality nucleic acids.

Several commercially available kits were purchased in order to assess their capacity to isolate RNA (and DNA) in terms of yield and amplifiability. The RNA (and DNA) extracted by these kits was compared to parallel samples extracted via TRIzol®. The High Pure RNA Isolation Kit (Roche), AquaRNA Kit (MultiTarget Pharmaceuticals), AllPrep DNA/RNA Mini Kit (Qiagen) were used according to manufacturer's recommendations. The highest level of HBB was detected in the TRIzol® sample followed by AllPrep, AquaRNA and HighPure (Figure 8).

Since the AquaRNA and AllPrep kits are recommended for extraction of DNA as well, we compared DNA yields to TRIzol® and the validated DNA extraction method used by the Vermont Forensic Laboratory for casework samples ("Organic"). Figure 9 shows that the AquaRNA kit is not useful for DNA extraction. Although the DNA yield from the AllPrep kit is significantly less than that of the Organic method, it is comparable with the TRIzol® method. Therefore, the AllPrep kit was deemed suitable for extraction of both DNA and RNA.

Since one of our long-term goals is to develop identification assays for human tissues, we sought an extraction technique which would work on tissue samples. The Absolutely RNA® Miniprep

Kit (Stratagene) was one option which we hoped would work without mechanical homogenization; an uncommon procedure in most forensic labs and therefore would require the purchase of new equipment. Before we attempted to work with human tissues, a comparison with the AllPrep Kit was performed using dried seminal fluid. As shown in Figure 10, both the Absolutely RNA® and AllPrep Kits were able to extract RNA from semen stains.

Once the Absolutely RNA® Kit was shown to work with simple stains, we attempted to extract RNA from various human tissues: kidney, adipose, colon and skin. Approximately 10-20 mg of tissue was used which had previously been stored at -20˚C for ~10 months following collection. Following reverse transcription, the samples were amplified using the B2M control TaqMan® assay. For comparison, parallel samples used QIAgen's QIAshredder columns in conjunction with the Absolutely RNA® Kit. However, the QIAshredder columns did not have a significant impact on the yields (data not shown), and therefore were not used in subsequent extractions of human tissues. All four tissue types tested were successfully amplified with the B2M control TaqMan® assay (Table 2).

In the search for an extraction method that would work for RNA and DNA, we came across a paper which outlined a simultaneous extraction method for both nucleic acids. Published by Berendzen et al. (2005), a method utilizing a sucrose buffer was reported to yield sufficient quality and quantity of RNA and DNA from plants. We first compared this method with our standard TRIzol® protocol to determine its capacity to extract RNA and DNA from blood (Figure 11).

Although the Sucrose method didn't appear to be an efficient RNA extraction method, we were surprised at how well it performed DNA isolation. A 10 µl blood spot extracted by Sucrose yielded 2.1 ng/µl DNA, versus 0.11 ng/µl from a parallel stain extracted with TRIzol®. However, throughout our studies, TRIzol® has never proven to be the best method for DNA extraction. Therefore, as a side investigation unrelated to the goals of the grant, we performed a direct comparison with our in-house "gold standard" Organic DNA extraction method, as well as with the Qiagen BioRobot® M48 which we were using for CODIS samples at the time. DNA was isolated from blood and buccal cells spotted on FTA® paper (Whatman, Florham Park, NJ); five millimeter diameter punches from each blood and buccal cell sample taken were used for the extractions. Twelve DNA databank samples were extracted by each method (i.e. Organic-blood, BioRobot® M48-blood, Sucrose-blood, Organic-buccal, BioRobot® M48-buccal, Sucrose-buccal).

The purpose of this experiment was to determine if the Sucrose Method is capable of extracting comparable amounts of DNA from samples as the Organic and BioRobot® M48 Methods. Convicted offender samples consisting of either dried blood or buccal cells on FTA® paper were examined. The data in Table 3 shows that there were no significant differences between the DNA yields from the three methods. Importantly, the Sucrose Method yielded DNA of sufficient quantity (greater than 0.1 ng/µl DNA) to perform downstream STR analysis. In our initial sucrose studies, in DNA extracts that required less than a 3-fold dilution (i.e. below 0.3 ng/µl) prior to DNA amplification for STR analysis, there was a decreased success rate for STR analysis due to an as yet unknown inhibitor present in the extracts (data not shown). Therefore, we added a clean-up step to the procedure which uses Millipore MultiScreen PCR vacuum filter

plates. This additional step circumvents downstream STR failures arising from the inhibitor (results not shown).

The amount of DNA recovered from all samples was sufficient for further DNA analysis. Thirty-six blood and 36 buccal cell samples (12 per extraction method) were analyzed with the Identifiler™ PCR Amplification Kit. Seventy-one samples were diluted to 0.1 ng/µl and 10 µl (1.0 ng total) was used for Identifiler™ STR analysis. One blood sample extracted using the Organic Method was used neat because the concentration was below 0.1 ng/µl. In order for the STR profiles to be deemed acceptable, they were evaluated under the following laboratory criteria: (1) heterozygous peak heights must be between 150 and 5500 relative fluorescence units (RFUs), (2) homozygous peak heights must be between 300 and 5500 RFUs, and (3) for heterozygous loci, the peak height ratios cannot be less than 60%.

Table 4 shows the results for two selected loci representing small and large amplicons (*D3S1358* and *CSF1PO*, respectively). All thirty-six of the blood samples yielded full STR profiles. Thirty of the buccal cell samples yielded full STR profiles while 6 samples yielded only partial profiles based on the criteria listed above. One Organic, three BioRobot® M48, and two Sucrose samples had either (1) heterozygous peak heights less than 150 RFUs, (2) homozygous peak heights less than 300 RFUs, and/or (3) heterozygous peak height ratios below 60% at one or more loci. Taken together, these results imply that the Sucrose Method is comparable to the Organic and BioRobot® M48 methods for extracting DNA of sufficient quantity and quality to perform STR profiling.

Since Berendzen et al. (2005) originally reported this method as a means to simultaneously extract DNA and RNA we performed exhaustive studies to determine whether RNA extraction via the Sucrose Method was possible since the DNA extraction was shown to work so well. The published procedure recommended a 10 minute incubation with the sucrose solution at 100˚C. We varied the length of incubation between 0 and 20 minutes which only worsened the RNA yields as determined by amplification with the HBB TaqMan® assay. The best condition remained the 10 minute incubation. On the other hand, the best DNA yields were in samples incubated for 20 minutes; however, any length of time greater than 5 minutes yielded sufficient amounts of DNA to perform downstream analysis (i.e. STR profiling).

Our next series of experiments focused on the addition of RNase inhibitors to the samples or changing the conditions under which the sucrose solution was added to the sample. In the first experiment, the sucrose solution was added to the stain either at room temperature (as done previously), or preheated to 100˚C in an attempt to inactivate RNases. Furthermore, 1 U/µl of RNase inhibitor was added to the sucrose solution immediately before use. Interestingly, the best amplification with the HBB TaqMan® assay was with the standard extraction conditions, namely room temperature sucrose solution and no RNase inhibitor. But, the real-time results were roughly 5 Cts less than what is normally seen following TRIzol® extraction of comparable samples. Neither of these permutations had a significant effect on the DNA yields.

Another experiment took a closer look at the temperature during the incubations: 56˚C, 75˚C and 100˚C. At the same time, a new RNase inhibitor, SUPERase·In (Ambion), was added to the sucrose solution. Again, the best condition for RNA extraction was at 100˚C, similar to DNA. The reagent RNA*later*™ (Sigma, St. Louis, MO) was used next in an attempt to protect the

RNA. RNA*later*™ is a storage reagent that rapidly permeates tissue to stabilize and protect cellular RNA without jeopardizing the quality or quantity of RNA. Normally, this reagent is used in conjunction with isolation of RNA from tissues, but we were hoping it might also work under our conditions. RNA*later*™ was added at one of 3 different steps of the procedure; 1) before the addition of sucrose solution, 2) with the sucrose solution, and 3) just prior to storage. Unfortunately, the RNA*later*™ made it worse; no amplification occurred with the HBB TaqMan® assay under any condition where RNA*later*™ was used. The same held true for the DNA samples; 10.7 ng/µl of total DNA was detected under standard extraction conditions, whereas absolutely nothing was detected in samples treated with RNA*later*™. Since RNA*later*™ seemed to be causing so many problems with the real-time assays we split the final sucrose extract into two portions (RNA, DNA) and added the reagent only to the RNA fraction. Although the DNA yields remained high, the RNA was still significantly below yields generated with more robust extraction methods (i.e. TRIzol®).

The sucrose solution was pretreated with RNA*secure*™ (Ambion) immediately prior to extraction since it has the capacity to inhibit RNase activity. The sucrose solution and RNA*secure*™ were mixed and heated to 60˚C for 10 minutes before use. As shown in Figure 12, the addition of RNA*secure*™ increased the Cts. One last ditch effort that we made to save the sucrose method as a dual extraction technique was the addition of proteinase K to the sucrose solution. However, since proteinase K is inactivated at 100˚C, the 10 minute incubation had to be performed at 56˚C to maintain the integrity of the enzyme. Figure 12 shows that the proteinase K, alone or in conjunction with RNA*secure*™ pretreatment of the sucrose solution, significantly decreased the amplification of the HBB TaqMan® assay. Interestingly, the

treatment of sucrose solution with RNA*secure*™ did not affect the DNA yield, as was seen with RNA*later*™. The conditions used with proteinase K also had no effect on the DNA yields.

Unfortunately, all of the measures that we attempted to increase the quantity and/or quality of RNA extracted via the sucrose method were unsuccessful. However, through these experiments we were able to develop, optimize and validate a new DNA extraction technique which has led to significant savings in terms of analyst time and reagent costs. Also, we were able to rule out a number of commercial kits that were recommended extraction methods. Future studies of this grant utilized the TRIzol® method (for stains) and Absolutely RNA® Kit (for tissues) for RNA extraction, as well as the AllPrep Kit for dual extraction of RNA and DNA.

Aim #2: To determine the half-life of mRNA in different types of stains under different conditions.

Prior to and throughout the course of the grant we have been collecting samples in order to determine the stability of candidate mRNAs for stain identification. In order for a candidate to be implemented into a stain identification assay, it needs to be detectable for years following deposition of the sample. In most cases, RNA was extracted with TRIzol® unless noted. To determine the amount of amplifiable RNA, we performed real-time PCR using HBB (blood), PRM2 (seminal fluid) or the housekeeping gene B2M.

One of the earliest experiments we performed was to see whether amplifiable RNA could be detected from an aged blood sample. From preliminary studies, we had 20 µl and 40 µl blood stains on cotton cloth which had been stored at room temperature in the dark for 13 and 26 months, respectively. Following amplification with the TaqMan® HBB assay, it was apparent

that this candidate gene was very stable over time (Figure 13). Similar results were seen for detection of the candidate gene PRM2 in seminal fluid. Twenty µl seminal fluid stains aged for 16 or 24 months were easily detected using this assay (Figure 14).

Now that we had determined the HBB and PRM2 genes were amplifiable in samples aged for two years, we performed time course studies to look at the stabilities over time. For blood, we used two sample volumes, 1 µl and 10 µl that had been aging at room temperature in the dark for 3 days to 510 days (1 µl) or 864 days (10 µl). As depicted in Table 5, there is a slight decrease in expression over time, but samples aged well over a year (or more) maintained a high HBB expression level.

In a similar experiment, we looked at two different sized seminal fluid stains, 1 µl and 20 µl that had been aging at room temperature in the dark for 16 days to 807 days (1 µl) or 791 days (20 µl). Table 6 shows that PRM2 expression is maintained in samples aged over 2 years. In fact, it appears as though the expression may even increase over time, perhaps due to the loss of an inhibitor.

The feasibility of extracting RNA from aged samples using methods other than the TRIzol® reagent was investigated. Both the AllPrep and Absolutely RNA® Kits were used to extract various amounts of aged blood and seminal fluid stains. In the first experiment, between 1 and 20 µl of blood aged 759 days was extracted using the AllPrep Kit and RNA samples were amplified using the HBB TaqMan® assay. The AllPrep Kit was also used to extract aged seminal fluid samples. Cuttings ranging from 1x1 mm to 5x5 mm (aged 1068 days) were extracted and analyzed with the PRM2 TaqMan® assay. Figures 15 and 16 demonstrate that the

AllPrep Kit is a suitable method for RNA extraction, since we were able to amplify HBB and PRM2 at levels comparable to what's been shown for TRIzol® extracts.

In a subsequent experiment, 20 µl of blood (aged 1031 days) was extracted using the Absolutely RNA® Kit and following reverse transcription; the cDNA was amplified using the HBB TaqMan® assay yielding a Ct of 20.92. The Absolutely RNA® Kit was also used to extract aged seminal fluid samples. Cuttings ranging from 1x1 mm to 5x5 mm (1068 days) were extracted and analyzed with the PRM2 TaqMan® assay. Figure 17 together with the blood result demonstrates that the Absolutely RNA® Kit is a suitable method for RNA extraction, since we were able to amplify HBB and PRM2 at levels comparable to what's been shown for TRIzol® extracts.

To demonstrate the stability of RNA over time, we extracted RNA from the first sample collected for this project; a seminal fluid stain aged for 1666 days at room temperature in the dark. Parallel 5x5 mm cuttings were extracted by TRIzol® or the AllPrep Kit. The resulting RNA was reverse transcribed and analyzed with the PRM2 TaqMan® assay. Figure 18 shows the stability of PRM2 RNA expression over time as well as the ability of both kits to extract RNA from aged forensic stains.

Forensic samples are often exposed to a variety of environmental insults therefore, we stored parallel samples under ideal conditions (room temperature, dark) as well as in a sub-optimal condition (37˚C). By comparing gene amplification between the samples, we hoped to determine whether RNA profiling would be feasible for weathered forensic stains. In the first experiment, between 0.25 µl and 20 µl of blood was spotted onto cloth and dried at either room

temperature in the dark, or in a 37˚C incubator. The samples were stored for 503 days prior to extraction with TRIzol®. cDNA was amplified using the HBB TaqMan® assay. Figure 19 shows that amplification of HBB is possible following storage at 37˚C for 503 days. In fact, there does not appear to be a considerable decrease in the degree of amplification after prolonged exposure to elevated temperatures.

One experiment we performed was to evaluate blood samples which had been stored under both conditions (room temp/dark, 37˚C) for an extended period of time with a relatively new sample. One µl blood stains were stored for 544 days at room temperature (dark) or in a 37˚C incubator and extracted with TRIzol®. For comparison purposes, a 1 µl blood stain was stored for 3 days at room temperature (dark) in order to see how much amplification of HBB decreases with prolonged storage at either of the two conditions. Regardless of the storage length or conditions, the resulting Cts were essentially the same with a difference of less than 1 Ct between all 3 samples (Figure 20). This result further demonstrates how stable HBB RNA is over time.

In a similar experiment, between 0.5 µl and 20 µl of semen was spotted onto cloth and dried at either room temperature in the dark, or in a 37˚C incubator. The samples were stored for 518 days prior to extraction with TRIzol®. cDNA was amplified using the PRM2 TaqMan® assay. Figure 21 shows that amplification of PRM2 is possible following storage at 37˚C for 518 days. Similar to what was shown for blood and HBB expression, there does not appear to be a considerable decrease in the degree of amplification after prolonged exposure to elevated temperatures.

Aim #3: To find 2-3 genes specific for each of four stain types (semen, blood, urine, saliva).

Survey of literature including PubMed, Gene and other databases have allowed for the selection of several genes that appear to be specific for a number of different tissues (Tables 1a and 1b). The following alphabetical list is a brief description of each gene target utilized over the course of the project:

ACPP (acid phosphatase, prostate) - enzyme which catalyzes the conversion of orthophosphoric monoester to alcohol and orthophosphate; secreted by the epithelial cells of the prostrate gland

ADCY1 (adenylate cyclase 1, brain) - encodes a form of adenylate cyclase expressed in brain

ALPI (alkaline phosphatase, intestinal) - digestive brush-border enzyme that is upregulated during small intestinal epithelial cell differentiation

ANKRD15 (ankyrin repeat domain 15) - protein containing four ankyrin repeat domains in its C-terminus and suggested to have a role in tumorigenesis of renal cell carcinoma

ANXA13 (annexin A13) - role in the regulation of cellular growth and in signal transduction pathways

AQP6 (aquaporin 6, kidney specific) - functions as a water channel in cells

B2M (beta 2 microglobulin) - serum protein found in association with the major histocompatibility complex class I heavy chain on the surface of nearly all nucleated cells

CD3G (CD3G antigen, gamma polypeptide) - component of the T-cell receptor-CD3 complex

CDH16 (cadherin 16, KSP-cadherin) - functions as the principal mediator of homotypic cellular recognition, playing a role in the morphogenic direction of tissue development

CLCNKA (chloride channel Ka) - functions in salt reabsorption in the kidney and potassium recycling in the inner ear

CPT1A (carnitine palmitoyltransferase 1A) - initiates the mitochondrial oxidation of long-chain fatty acids and is the key enzyme in the carnitine-dependent transport across the mitochondrial inner membrane

CRISP1 (cysteine-rich secretory protein 1) - expressed in the epididymis and plays a role at fertilization in sperm-egg fusion

DCXR (dicarbonyl/L-xylulose reductase) - enzyme that has both diacetyl reductase and L-xylulose reductase activities

DRD1 (dopamine receptor 1) - G-protein coupled receptor stimulates adenylyl cyclase and activates cyclic AMP-dependent protein kinases; regulate neuronal growth and development, mediate some behavioral responses, and modulate dopamine receptor D2-mediated events

FABP2 (fatty acid binding protein 2, intestinal) - participates in the uptake, intracellular metabolism and/or transport of long-chain fatty acids

GAPDH (glyceraldehyde-3-phosphate dehydrogenase) - catalyzes an important energy-yielding step in carbohydrate metabolism, the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and nicotinamide adenine dinucleotide

GAPDHS (glyceraldehyde-3-phosphate dehydrogenase, spermatogenic) - plays an important role in regulating the switch between different energy-producing pathways, and it is required for sperm motility and male fertility

GPM6A (glycoprotein M6A) - stress-responsive gene involved in hippocampal formation

GYS2 (glycogen synthase 2) - catalyzes the formation of α-1,4-linkages that elongate chains of glucose molecules to form glycogen

HBB (hemoglobin, beta) - iron containing oxygen-transport metalloprotein in red blood cells

HTN3 (histatin 3) - non-immunological, anti-microbial role in the oral cavity

LEAP2 (liver expressed antimicrobial peptide 2) - cysteine-rich cationic antimicrobial peptide

LIPC (lipase, hepatic) - dual functions of triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake

MIOX (myo-inositol oxygenase) - key regulator of inositol levels, catalyzing the first committed step in the glucuronate–xylulose pathway

MSMB (microseminoprotein, beta) - synthesized by the epithelial cells of the prostate gland and secreted into the seminal plasma

MYBPC3 (myosin binding protein C, cardiac) - cardiac isoform of myosin-binding protein C; a myosin-associated protein found in the cross-bridge-bearing zone (C region) of A bands in striated muscle

PFKL (phospho-fructokinase, liver) - catalyzes the conversion of D-fructose 6-phosphate to D-fructose 1,6-bisphosphate in glycolysis

PRM2 (protamine 2) - major DNA-binding protein in the nucleus of sperm and packages DNA

PSA (kallikrein 3) - protease present in seminal plasma involved in liquefaction of seminal coagulum

PYGL (phosphorylase, glycogen) - catalyzes the degradation of glycogen to glucose-1-phosphate by the phosphorylytic cleavage of a 1,4-glycosidic bond

REN (renin) - catalyzes the first step in the activation pathway of angiotensinogen; a cascade that can result in aldosterone release, vasoconstriction, and increases in blood pressure

RYR2 (ryanodine receptor 2, cardiac) - one of the components of a calcium channel, composed of a tetramer of the ryanodine receptor proteins and a tetramer of FK506 binding protein 1B proteins, that supplies calcium to cardiac muscle

SEMG1 (semenogelin I) - involved in formation of gel matrix that encases ejaculated spermatozoa

SPTB (spectrin, beta, erythrocytic) - major component of red blood cell membrane

STAT (statherin) - peptide that inhibits precipitation from calcium phosphate solutions, stabilizes saliva

TCF2 (transcription factor 2, hepatic) - encodes a member of the transcription factor superfamily which forms heterodimers with another member of this transcription factor family, HNF1A

TGM4 (transglutaminase 4) - catalyzes the cross linking of proteins and the conjugation of polyamines to specific proteins in the seminal tract

TNNT2 (troponin T type 2, cardiac) - tropomyosin-binding subunit of the troponin complex, which is located on the thin filament of striated muscles and regulates muscle contraction in response to alterations in intracellular calcium ion concentration

UPK2 (uroplakin 2) - believed to strengthen the urothelial apical surface and prevent the cells from rupturing during bladder distention

Applied Biosystems has created TaqMan® primer/probe sets for many human genes (Assay-on-Demand™ products). These have been carefully designed and thoroughly tested for specificity. Most of these sets are mRNA specific (cross exon-exon boundaries and have no cross-reaction with pseudogenes) although some do react with genomic DNA. Since these sets are available, optimized and cost only $150 each, we decided to use these pre-designed sets for our initial studies rather than expend the time and resources necessary to design our own. Table 1a lists the

Assay-on-Demand™ numbers for each of the genes of interest. The mRNA specific assays (probe spans an exon/exon junction) end in "_m1". Those ending in "_g1" are not guaranteed to be mRNA specific. We checked the results when using two of the _g1 probes (HBB and PSA) by amplifying with DNA. Figure 22 shows the result of amplification of cDNA and genomic DNA (256 ng in a 10 ul reaction) with five TaqMan® assays; only the cDNA was amplified.

We tested the specificity, robustness and sensitivity of each assay by analysis of mRNA isolated from the body fluid of interest. Once the assay was been shown to be robust, we tested it on mRNA isolated from other fluids and tissues to demonstrate that the assay is specific. Additional fluids and tissues to those discussed previously included vaginal secretions and menstrual blood. For semen, we are interested in mRNAs specific for the sperm as well as for the prostatic components. In a number of cases, it is important to determine if semen is present even if the male is sterile or has had a vasectomy. It other cases, it is important to know if sperm are likely to be present. We obtained anonymous samples of a variety of tissues (kidney, colon, adipose, skin) from the Surgical Pathology Suite at Fletcher Allen Health Care. Lastly, control RNAs (liver, kidney, brain, heart, intestine) were purchased from Ambion to ensure that our assays are specific only for the intended tissue.

The sensitivity of the three candidate blood assays (HBB, CD3G, SPTB) was evaluated using a range of blood volumes spotted onto cotton cloth. Between 0.0001 µl and 20 µl of blood was allowed to air dry at room temperature for 18 hours prior to extraction using TRIzol®. cDNA was amplified using the three TaqMan® assays. The sensitivity of HBB was far superior to the other assays with amplification resulting from 0.0001 µl of blood, compared to a limit of 1 µl for the CD3G and SPTB assays (Table 7).

The sensitivity of the four candidate semen assays (SEMG1, PSA, MSMB, TGM4) and one sperm assay (PRM2) was evaluated using a range of semen volumes spotted onto cotton cloth. In one experiment (SEMG1, PSA, PRM2), between 0.01 µl and 20 µl of semen was allowed to air dry at room temperature for 8 days prior to extraction using TRIzol®. In a subsequent experiment (MSMB, TGM4), between 1 µl and 20 µl or semen was allowed to air dry at room temperature for 41 days prior to extraction using TRIzol®. cDNA was amplified using the five TaqMan® assays. The sensitivities of PSA and PRM2 were slightly higher than SEMG1 since both were able to amplify from 0.01 µl of semen (Table 8). However, since the Cts for PRM2 were significantly lower than those for PSA (and SEMG1) for most volumes, PRM2 appears to have a higher expression level, or is a more robust assay. Furthermore, the second experiment with MSMB and TGM4 suggests that those two candidates are expressed more than the other semen markers (SEMG1 and PSA) since the Cts are lower. But, since the two lowest volumes, namely 0.01 µl and 0.1 µl, were not analyzed with these two assays, the lower limits are unknown, but assumed to be lower than 1 µl based on the Cts.

In an attempt to get a better handle on the lower detection limits of the semen and sperm assays, we utilized a control testes sample from Panomics. Since this sample was of a known quantity we were able to generate a dilution series to test along with our TaqMan® assays. One µl of RNA from samples ranging from 1 ng/µl to 1 µg/µl was used in RT reactions to generate cDNA. The resulting cDNA was amplified with the following assays: SEMG1, MSMB, TGM4 and PRM2. Based on the results shown in Table 9, PRM2 has the highest expression level based on the Cts. Both PRM2 and TGM4 were detected in 1 ng of RNA, whereas MSMB and SEMG1 were detectable at 10 ng and 100 ng, respectively. Although our goal of developing screening

assays does not involve quantitation of our samples, this experiment demonstrated that TGM4 and PRM2 are robust candidates for the detection of semen or sperm, respectively.

The sensitivities for saliva candidate markers (STAT, HTN3) were evaluated using a range of saliva volumes spotted onto cotton cloth. Between 0.01 µl and 20 µl of saliva was allowed to air dry at room temperature for 18 hours prior to extraction using TRIzol®. cDNA was amplified using the two TaqMan® assays. As shown in Table 10, only the STAT assay was able to detect 1 µl of freshly dried saliva, whereas the HTN3 assay had a 5 µl limit of detection. The Cts were not as low as seen in the blood and semen/sperm assays, but were different than the absence of Cts observed for the negative controls.

Unfortunately the results from urine TaqMan® assays were not what we hoped for. Testing two potential candidates, REN and UPK2, a 20 µl urine stain dried for 18 hours was amplified only by the UPK2 assay. The Ct for this sample was 35.15; REN did not amplify in this sample. Therefore, other potential candidates need to be identified for urine.

The specificity of the chosen genes for the tissue of interest was also examined. To test the TaqMan® sets (only the control B2M and tissue-specific sets should give amplification), mRNA was isolated from dried blood, semen, saliva, menstrual blood or vaginal secretions using TRIzol®. Human tissues (kidney, colon, adipose, skin) were extracted using the Absolutely RNA® Kit. Control human RNAs from Ambion (brain, heart, liver, kidney, intestine) were diluted to 100 ng/µl. The RNA samples were then reverse transcribed and PCR performed with each of the TaqMan® sets. The amplification results for the blood, semen, sperm, and saliva assays are depicted in Table 11.

For the three blood assays, amplification only occurred in the blood sample as expected. For the 6 semen assays there were mixed results. PSA, SEMG1, MSMB and TGM4 all appeared to be specific for semen when tested against blood, semen and saliva. However, SEMG1 cross-reacted with vaginal secretions, albeit at a much lower level (Ct of 29.40 for semen vs. Ct of 33.67 for vaginal secretions). Interestingly, MSMB amplified from a control intestine sample, but not with human colon tissues. ACPP and CRISP1, although ideal candidates based on literature searches, were not suitable assays for the specific detection of semen. The sperm marker PRM2 amplified from a seminal fluid sample, but nothing else that was tested. For saliva, amplification only occurred with the STAT assay as expected. The control B2M was detected in each of the fluids and tissues. Based on these results, several of these TaqMan® assays appear to be specific (HBB, SPTB, CD3G, PSA, STAT), however, screening against an expanded sample set would strengthen that claim. Alternatively, TGM4 and PRM2 have demonstrated their specificity for semen/sperm through the course of these studies.

The specificity studies using the tissue assays yielded mixed results (Table 12). One of the brain assays (GPM6A) only amplified from the control brain sample as expected, but the other two also cross-reacted with other control tissues. DRD1 had a Ct value of 25.31 with brain, but Cts of 28.63 and 29.78 with liver and intestine, respectively. ADCY1 had a Ct value of 19.88 with brain, but Cts of 23.60 and 23.99 with heart and kidney, respectively. The Cts for ADCY1 and GPM6A (20.55) were the lowest for brain samples out of the three assays which indicates that they are expressed to a higher extent than DRD1.

Using control RNAs and three fluids, the heart TaqMan® assays were very specific for their intended target. The only sample that showed amplification with the three assays was control

brain RNA. Similarly, the intestine assays were very specific when screened against the control RNAs, blood, semen and saliva. However, all three amplified from kidney as well as colon tissue. Furthermore, the expression between the two tissue types appeared to be equivalent for the three targets. The Cts for ANXA13, ALPI and FABP2 were nearly the same when tested against human kidney and colon samples.

Finding tissue-specific markers for liver and kidney was the most challenging. Table 13 shows the number of candidates that were evaluated. To date, we have screened 7 different liver markers and 6 kidney markers. Both PFKL and CPT1A amplified with all 5 control RNAs that we utilized in the project. TCF2, PYGL and LEAP2 amplified with 3 or 4 of the control RNAs. PYGL also amplified in a blood sample. However, the Ct for PYGL in the liver sample was 24.84, whereas for blood and the other control RNAs, the Cts were greater than 27.6. Only LIPC and GYS2 showed the potential to be tissue-specific. Although LIPC was amplified using both liver and kidney samples, there was a significant difference in the expression levels. The liver sample yielded a Ct of 18.85, whereas the kidney sample yielded a Ct of 23.06. GYS2 was only amplified using the liver samples. However, before any claims can be made regarding the specificity of these two assays, experiments using actual human tissues (liver) need to be performed. Unfortunately, we have not been able to obtain human liver samples through our collaborators to date.

Of the 6 kidney TaqMan® assays which have been evaluated, one (AQP6) failed to amplify from any of the experimental control RNAs, and three others (CDH16, DCXR, ANKRD15) amplified from all 5 of the control samples. However, CDH16 had been identified as a potential intestinal marker through these studies. The Ct for CDH16 with control intestine RNA was 17.89, whereas

the other samples had Cts of 28.48 or higher. Using the human tissues, both kidney and colon were detected; kidney with Cts ranging from 30.81 to 35.43 and colon with Cts of 23.82 and 30.45. MIOX appears to be a specific marker for kidney. Both control kidney RNA and RNA extracted from 4 individual kidney donors were amplified with this assay. Furthermore, there was no cross-reactivity with any of the other samples tested. The only two sources not tested were menstrual blood and vaginal secretions which are unlikely to yield a positive result, but remain to be tested.

Aim #4: To multiplex the assays developed for the detection of stain types.

A major aim of stain identification using mRNA expression profiling is working towards multiplexing the real-time PCR assays once mRNAs are identified that clearly define specific types of stains. Since these assays are designed to function more qualitatively than quantitatively, a test of a single stain should typically give amplification with only one candidate. It is possible that a mixture could give several amplifications. However, it is of greater concern to know that a mixture does exist, than to know the exact amount of each fluid present.

Plexor® Analysis

One methodology to achieve this goal is the Plexor® system from Promega. Depending on the dye-capability of the real-time instrument that is utilized, this system allows multiple mRNAs to be combined in one assay, thus reducing the amount of sample needed and time of analysis. For example, using a 4-dye channel instrument, eight mRNA targets could be analyzed; two per dye-channel, each of which has a distinctive melt curve. The Plexor® qRT-PCR system takes

advantage of the specific interaction between two modified nucleotides to achieve quantitative PCR analysis (Figure 2). Promega's Plexor® design software allows the generation of primers and probes which span an intron by designating a certain base to include in the primer/probe design.

Our initial focus was to design a blood-semen stand alone assay, since our initial studies have identified mRNAs that are specific for these fluids. We designed Plexor® primers to simultaneously detect HBB, CD3G, PSA and PRM2 in order to identify whether blood and/or semen is present is a stain. In order to determine whether the technology would work in our hands with forensic samples, only HBB and PSA were ordered. HBB was labeled with FAM and PSA was labeled with CAL Orange 560 (i.e. HEX). RNA was extracted from 1 µl blood or semen stains using TRIzol®. The HBB and PSA Plexor® sets were first tried in singleplex reactions using 2 different concentrations of primers (100 nM and 200 nM). Both concentrations of primers amplified from the blood sample, as expected, with no amplification in the no template controls. The reaction using 200 nM of primers had a significantly lower Ct than the reaction with 100nM of primers (30.6 vs. 36.9). The PSA primers amplified from the semen samples, but the Cts were equivalent to the no template control samples. We believed that we were seeing primer-dimer interactions with the PSA primers, so we changed the annealing temperature from 60˚C to 65˚C, decreased the number of cycles from 40 to 37, and used 150 nM of primers. While the HBB primers seemed to work slightly better under these conditions (Ct of 28.3 vs. Ct of 30.6), we still saw amplification in the no template controls using the PSA primers. After speaking with technical support at Promega, it was recommended that we design new PSA primers.

The results using the newly designed PSA primers were not significantly better. In the first experiment with the new primers, there was a significant difference in Cts for the no template control and the semen sample (37.9 vs. 31.6). However, the No RT sample (lacking the RT enzyme) amplified with a lower Ct (30.3) than the sample with the enzyme. This suggests that there may be some amplification of genomic DNA even though the primers were designed to span an intron.

Our studies looking for tissue-specific genes identified PRM2 as a specific and robust sperm marker. As mentioned above, PRM2 was included in the original multiplex for a stand alone blood-semen assay. The primers for PRM2 were labeled with CAL Red 610 (ROX) and tested in monoplex reactions for the ability to detect RNA from seminal fluid stains. The results for the PRM2 Plexor® primers were the most successful to date. While no amplification occurred in the no template control sample, a Ct of 34.2 was generated for the No RT reaction. However, the reaction containing semen and the RT enzyme yielded a Ct of 23.7, nearly 10 Cts below the control sample.

A more thorough study to optimize the primer concentrations for HBB, PSA and PRM2 was performed in monoplex reactions. For the HBB primers, 150 nM yielded the lowest Ct (22.4), followed by 200 nM (23.2) and 100 nM (27.3). The negative controls were all as expected for the HBB primers and there was no cross-reactivity with semen samples. A concentration of 200 nM PRM2 primers yielded the lowest Ct at 36.3; not as low as seen in previous experiments, but less than the negative controls and blood samples which did not amplify. The ideal concentration of PSA primers was 200 nM followed by 150 nM and 100 nM. In this experiment,

there was a clear distinction between the Cts for the semen sample (Ct of 29.4) and the other negative controls (Ct >36.5) and blood samples (Ct > 37.5).

The final candidate for our blood-semen assays was the blood marker CD3G. We ordered this primer pair labeled with Quasar 670 (CY5). Unfortunately, the results using this primer pair were not as expected. In the first monoplex reaction we attempted, the Cts ranged from 7.3 to 31.6 with the lowest belonging to a No RT semen sample and the highest belonging to the semen sample with enzyme. Therefore, we didn't pursue the addition of this primer pair to the blood-semen assay.

In a multiplex reaction of HBB, PSA and PRM2 we demonstrated that amplification of each fluid only occurred with the tissue-specific genes as expected; blood was amplified with HBB primers, whereas only semen was amplified with PSA and PRM2 primers (Table 14). Additionally, an artificial mixture of blood and semen RNA extracts shows that these primer sets are able to discriminate their target RNA in a heterogeneous sample. Therefore, at this point in time we have utilized three-dye technology to multiplex blood and semen Plexor® primer sets into a single assay.

The initial success of the blood-semen assay using the Plexor® platform led to experiments to develop a quick, one-tube seminal fluid assay, since seminal fluid analysis is a major task for all forensic laboratories. For semen, we are interested in genes specific for sperm and prostatic components. Often, it is important to determine if semen is present even if the male is sterile or has had a vasectomy (i.e. no sperm). In other cases, it is important to know if sperm are present.

An assay that could determine whether the stain 1) is seminal fluid and 2) contains sperm could alleviate the need to perform extensive microscopy for the identification of sperm.

The original candidates tested in monoplex reactions for the seminal fluid assay were: ACPP (CAL Orange 560), PSA (CAL Orange 560), SEMG1 (CAL Red 610) and GAPDHS (FAM). However, since both ACPP and PSA were labeled with the sample dye, we would need to change the dye on one of the markers if they were shown to be useful in the assay. An initial experiment using 200 nM of primers at an annealing temperature of 62˚C for 35 cycles appeared to work well for all but the ACPP primer pair. The only detectable Cts were in the blood and semen samples, but unfortunately the blood Ct was nearly 20 Cts less than that for the semen. The results for PSA, SEMG1, and GAPDHS were as expected; the only significant amplification occurred in the semen samples. Interestingly, the most robust expression was seen with the GAPDHS primers (Ct of 24.7 vs. 30.6 and 30.4 for PSA and SEMG1, respectively).

With the various primer pairs that we had on hand it was possible to produce different multiplex sets by combining three different dyes which had been successful: CAL Orange 560 (PSA, ACPP), CAL Red 610 (SEMG1, PRM2) and FAM (GAPDHS). In the first triplex reaction, PSA (200nM), PRM2 (100nM) and GAPDHS (100nM) were combined. Whereas the PSA and PRM2 primers amplified from only semen samples, the GAPDHS primers showed amplification in a blood sample to a similar extent as the semen sample. A second triplex reaction of ACPP (200 nM), SEMG1 (200 nM) and GAPDHS (100 nM) was set up and fared no better. In this instance, ACPP primers showed the greatest degree of specificity for semen whereas the GAPDHS primers failed to amplify from a semen sample and the SEMG1 primers amplified from a negative control to the same extent as the semen sample. Therefore, under the conditions tested

to date, there was no clear indication of which primers which would perform consistently in a multiplex seminal fluid assay.

In an attempt to troubleshoot the multiplex of ACPP, SEMG1 and GAPDHS the primer concentrations were changed slightly so that all three were added at 200 nM. The GAPDHS primers amplified from the semen sample (Ct of 25.1) to a much greater degree than blood (Ct of 33.4) or the negative controls (Cts > 31.9). The only amplification observed from the ACPP primers was in the semen sample, although the Ct (32.0) wasn't as low as seen with GAPDHS. Similar to the previous experiment, amplification with the SEMG1 primers was no different in the semen sample than for the blood or negative controls. One last ditch effort was made to salvage SEMG1 as a semen target in Plexor® reactions. We kept the same primer sequences, but swapped the primer which had the fluorescent label attached, as recommended by Promega's technical support personnel. However, this new primer pair did not fair any better than the original pair. The Cts were equivalent regardless of which type of sample was analyzed: blood, semen, or the negative controls.

Based on the results of the previous two experiments, we decided to substitute PRM2 for SEMG1 in the seminal fluid assay. The primer concentrations were changed again in an attempt to compensate for the degree of expression between the three targets: 100 nM PRM2, 200 nM GAPDHS, 300 nM ACPP. Table 15 shows that this multiplex had a greater degree of success than the previous combination. All three primer pairs amplified from semen and the 1:1 mixture of blood and semen to a greater extent than blood alone or the negative controls.

In order to generate a multiplex consisting of four targets, we decided to order the PSA primers labeled with Quasar 670 (CY5), as opposed to the original label of CAL Orange 560 (HEX) since the ACPP primers are labeled with CAL Orange 560. In a preliminary study, we varied the concentration of PSA primers in the reaction from 50 nM to 500 nM. The greatest degree of amplification (i.e. the lowest Ct) occurred with a primer concentration of 500nM. Unfortunately, this Ct was not very different from the no RT control. We decided to DNase treat our sample in case we were seeing amplification of genomic DNA. A comparison of samples with and without DNase treatment showed that we were seeing a degree of genomic DNA amplification even though the primers were specifically designed to cross an intron (Table 16). In the sample treated with DNase, the difference in Cts between a No RT sample and the sample containing the RT enzyme is nearly 7 Cts. Alternatively, the same difference in a sample not treated with DNase is only 5 Cts. There was a slight decrease in amplification in the semen sample following DNase treatment which is likely due to a minor loss of RNA. However, DNase treatment of the No RT sample results in amplification similar to what's observed in the no template control sample. These finding suggests that DNase treatment may reduce some of the amplification seen in the No RT samples, in addition to requiring more time and effort to an assay which is designed to be a quick screening tool.

The PSA primer set labeled with CY5 was added to the seminal fluid triplex of GAPDHS, ACPP, and PRM2 to generate an assay with two semen-specific and two sperm-specific markers. As shown in Table 17, the multiplex results are promising. Although there was not a significant difference in Cts between the no template control and semen samples for GAPDHS, further optimization of primer concentrations may address that issue.

While we were in the process of optimizing our Plexor®-based multiplex assays, we were contacted by Promega with an offer to collaborate on the development of stain identification assays. The combination of Promega's extensive knowledge of the Plexor® platform with our sample inventory and insight into the needs of the forensic laboratory, it was a strong partnership. The goal was to generate Plexor® Tissue Typing Systems; multiplexed qRT-PCR systems for determining the source and quantity of a variety of human stains and/or tissues. The systems would include Plexor® primers for the detection of tissue-specific mRNA transcripts associated with semen, sperm, blood, menstrual blood, saliva, etc. By limiting the initial system to two-color detection (i.e. FAM and HEX detection), it would be compatible with the majority of real-time thermal cyclers in forensic laboratories. We sought to explore the potential to include controls (e.g. a housekeeping gene) or multiple, tissue-specific targets using the ability of the Plexor® Data Analysis Software to distinguish two different amplicons in the same dye channel, based upon thermal melt properties.

The Stain ID assay which we decided to first focus on was for detection of semen and saliva. Plexor® primers were designed to amplify the targets SEMG1 (semen), HTN3 (saliva) and GAPDH (housekeeper). Table 18 shows amplification of a variety of RNA samples using the triplex. The saliva primers (HTN3) show specific reactivity with saliva RNA (isolated in-house), and also with submaxillary gland RNA and salivary gland RNA that was purchased from a commercial vendor (BioChain, Hayward, CA). They show no cross-reactivity with other RNA samples tested, or with genomic DNA. The semen primers (SEMG1) show specific reactivity with semen RNA that was isolated in-house, and did not amplify any of the other RNA samples tested. However, they do show some minor reactivity with genomic DNA. This is likely due to

some weak amplification across the intron in the genomic sequence. Therefore, new SEMG1 primers were ordered that would hopefully address the genomic DNA amplification.

The modified SEMG1 primers were screened for the possibility of amplifying genomic DNA. The new primers did indeed eliminate genomic DNA contamination, however there was now cross-reactivity with blood RNA. The initial blood sample isolated showed no reactivity with the SEMG1 primers (old or new), but a commercial blood sample did show amplification with the SEMG1 primers. To confirm this, two additional blood RNA samples were generated in-house and tested; both of those showed some amplification with the SEMG1 primers as well (Figure 23). The blood amplification did not appear to be an issue with genomic amplification, since the melt curve is specific to the RNA product and not the genomic product. Also since the modified primers eliminated the amplification from the genomic DNA sample, but not from the blood sample. Therefore, we assume that SEMG1 is expressed at low levels in blood. Although the article doesn't mention blood specifically, Lundwall et al. (2002) reported SEMG1 to be expressed in a number of non-genital tissues. As a result of these findings, we decided to replace SEMG1 with a different semen-specific target.

Protamine 2 (PRM2) was identified as a potential target in early experiments, so primers were designed and screened for PRM1 and PRM2 transcripts. The PRM1 primers were designed so that one primer sits on the junction of the two exons, however some amplification of genomic DNA was detected. Three different sets of PRM2 primers were designed. The first set were designed so that one primer sits on the junction of two exons, but some amplification of genomic DNA was detected, as well as amplification from other RNA transcripts. The second set were designed so that one primer is on either side of the intron. Some amplification of genomic DNA

was detected; however, the DNA melt curve was distinct from the RNA melt (84˚C for RNA vs. 88˚C for DNA). The third set of PRM2 primers were designed so that one primer is on either side of the intron. Some amplification of genomic DNA was detected, however, the DNA melt curve was distinct from the RNA melt. Unfortunately, minor amplification of skin and blood also occurred with these primers. The second set of PRM2 primers looked the most promising although in theory, PRM2 is a sperm-specific marker and therefore, may not be expressed in vaseoctomized males. We isolated RNA from a semen sample donated by a vasectomized male and not surprisingly, PRM2 was not detected (Figure 24). At this point, because the screening assay only includes one semen/sperm marker, it was decided that PRM2 would not be a viable candidate since the presence of semen would go undetected in samples from vasectomized males or donors with low-sperm counts.

The next semen-specific candidate which was assessed was PSA. Initial studies with PSA primers showed non-specific amplification with all other RNAs that were tested, as well as genomic DNA and the no template control. Since all nonspecific amplification (including the no template control) was identical in the amplification and melt curves the cause was thought to be primer dimerization. However, additional experiments with variations in the PSA primer sequences yielded little improvement in the target specificity. Based on studies we had performed using the Gene Expression TaqMan® assays from Applied Biosystems, we switched our focus to MSMB and TGM4. Expression of MSMB occurred in both vasectomized and non-vasectomized semen samples, however, nonspecific amplification was observed with all the other RNAs as well as genomic DNA. Changing the fluorescent label to the opposite primer showed no improvement in target specificity. On the other hand, TGM4 was detected in both

vasectomized and non-vasectomized semen samples with no nonspecific amplification (Figure 25).

The preliminary experiments were not performed using sample types and sizes which are reflective of forensic-type samples. The RNA samples which were extracted in-house included vaginal secretions, whole blood, semen and saliva. In all cases, RNA was extracted from extremely large sample sizes (i.e. 2 mL of semen, 10 mL of saliva, 5 mL of whole blood) and the samples were all fresh. These were obviously not reflective of actual casework samples, but intended to isolate a large pool of RNA that experiments could be performed with. The semen, saliva and whole blood RNA samples were extracted with phenol-chloroform and the vaginal secretion was extracted using Promega's PureYield RNA midiprep kit. After extraction, the RNA was quantitated and 100 ng of RNA was used per reaction. Titration experiments were performed and indicated that semen had a detection threshold of approximately 10 pg of RNA whereas the threshold for saliva was approximately 1 ng of RNA. Although these studies gave an idea as to the relative sensitivities of the assay, it was important to assess how the assay would perform with typical forensic samples (i.e. various volumes of unknown RNA yields).

A panel of samples was screened using the Stain ID assay which now consisted of HTN3, TGM4, and GAPDH. Samples included dried blood (20 µl aged 70 days), semen (1-20 µl aged 76 days), saliva (1-20 µl aged 4 days), menstrual blood (5x5 mm cutting aged 700 days) and vaginal secretions (1/2 swab aged 54 days) extracted by TRIzol®. Positive control samples of known RNA quantities were run along with the Plexor® Stain ID assay: semen (1 ng/reaction) and saliva (10 ng/reaction). The results are shown in Table 19. The HTN3 primers amplified from all four saliva samples ranging from 1-20 µl of dried saliva as well as the positive control

sample. However, there was some nonspecific amplification in the vaginal secretions sample. The TGM4 primers only amplified from the semen samples and positive control. Interestingly, GAPDH was not amplified in the four saliva samples even though HTN3 was clearly detected which suggests that the expression of the housekeeper in saliva is not as prevalent as the tissue-specific marker. Based on the HTN3 results, the primers were modified by moving the fluorescent label from one oligo to the other. The modified HTN3 primers no longer detect vaginal secretions (Figure 26).

A major selling point of the Stain ID assay was the development of a co-isolation method for RNA and DNA extraction. By utilizing one extraction step, a DNA sample would be ready and waiting for STR profiling if the RNA screening assay deemed it worthy of such analysis. In addition, obtaining RNA and DNA from a single stain would prevent the possibility of conclusions being drawn regarding the identity of one stain which may not hold true for a nearby stain. Therefore, a significant amount of time was spent optimizing a procedure which would co-extract the two nucleic acids so that they were of sufficient quality and quantity for downstream analyses.

An initial comparison of DNA yields using two different extraction techniques was made. One and ten µl of semen and saliva were extracted via the RNagents Total RNA Isolation System (Promega) or an organic method which used Tris-buffered phenol. Prior to extraction with these methods a pre-processing step consisting of incubation with 1.8 mg/mL proteinase K and 100 mM DTT at 56˚C for 2 hours was performed. In parallel samples, this step was omitted and the samples went immediately into lysis with the guanidine denaturation solution. DNA yields were determined using the Plexor® HY quantitation assay. RNA yields were assessed using the

saliva-semen screening assay. The Tris-buffered phenol extraction provided the best results since the DNA yield was better than for the RNagents extraction while the RNA yields were equivalent (Table 20). The DNA yields were significantly higher when the pre-processing step was included in the protocol, however the RNA was virtually destroyed and undetectable in the Stain ID assay (Table 21).

Experiments were undertaken to improve the sample lysis without compromising RNA integrity. Reducing agents were added to the guanidine-HCl denaturation solution which itself should protect the RNA. A protocol was optimized which yielded sufficient quality and quantity DNA and RNA for downstream analyses. The detailed procedure is described below:

To the sample, 100 µl of RNagents denaturation solution, 90 µl of 1X PBS and 10 µl of 20 mg/ml proteinase K is added. The sample is vortexed and incubated for 10 minutes at 56°C. The sample is transferred to a spin basket and centrifuged at max speed for 2 minutes. To the flow-through, 20 µl of 2M sodium acetate is added and mixed followed by the addition and mixing of 12 µl of 1M Tris (pH 9.0). A volume of 230 µl phenol:chloroform:isoamyl alcohol is added and the sample vortexed and centrifuged at max speed for 5 minutes. The aqueous phase is removed into a new tube and 220 µl of chloroform:isoamyl alcohol is added and vortexed. The sample is centrifuged at max speed for 5 minutes and the aqueous phase is removed into a new 1.5 mL tube. Four µl of 5 µg/µl glycogen and 210 µl of isopropanol is added and vortexed. The sample is centrifuged at max speed for 10 minutes and following removal of the supernatant, the sample is washed with 1 mL of ice-cold 95% ethanol. Centrifuge at max speed for 5 minutes and remove supernatant and wash with 200 µl of 75% ethanol. Centrifuge at max speed for 5

minutes and remove supernatant and allow pellet to air dry for 1 minute. The pellet is resuspended in 30 µl of TE^{-4} and stored at -20°C.

The final optimized extraction method was used to extract 1 and 10 µl of semen and saliva. The resulting DNA was quantitated using the Plexor® HY assay with yields shown in Table 22. This extraction procedure yielded sufficient quantities of DNA to perform STR analysis. The RNA was used in the Stain ID assay to detect the presence of HTN3, TGM4 and GAPDH. The only amplification of HTN3 was in the saliva samples, whereas amplification of TGM4 only occurred with the semen samples. Alternatively, GAPDH was detected in both sample types. This experiment supports the use of Tris-buffered phenol extraction as a method to isolate sufficient quantities of quality nucleic acids for downstream analyses.

Promega is in the process of generating a draft Technical Manual that will accompany the Stain ID kit. Once that is finished, we will be alpha-testing the assay using 18 of our in-house samples. These samples include blood, menstrual blood, saliva, semen, urine, vaginal secretions, buccal swabs on FTA®, kidney, adipose, colon and skin. In addition, mixtures of blood/saliva, blood/semen, and saliva/semen will be tested. Multiple blood, saliva, semen and urine samples will be used that range in age and size. These samples have been extracted using the Tris-buffered phenol protocol outlined above and are awaiting the arrival of the Manual and final Stain ID amplification materials from Promega's R&D stocks. We plan to amplify the extracted total nucleic acid samples which will provide confirmation that the primer sequences are performing with the specificity we expect. The next step is to produce data for a validation paper using Stain ID materials that are made and QC'd by the manufacturing department at Promega. Once these are available, we will select and extract a larger sampling from our sample inventory

for validation. This second round of testing with the manufactured materials will include testing samples that are of different ages, spotted on different materials (i.e. other than cotton cloth), and stored under various conditions. Furthermore, these samples will also be evaluated for DNA yields and generation of STR profiles.

TaqMan® Analysis

As stated previously, one of the disadvantages to using the Gene Expression TaqMan® assays from Applied Biosystems is the inability to multiplex the assays since they are all labeled with the same dye. Therefore, we set out to design our own TaqMan® probes and primers in hopes of developing several specific multiplex assays. The first multiplex we designed was a semen-sperm detection assay using the TGM4 (CAL Orange 560; HEX) and PRM2 (FAM) markers in addition to the housekeeper B2M (CAL Red 610; ROX). Using the Beacon Designer program we designed a set of probes and primers which were ordered from Biosearch Technologies. Initially, experiments were conducted to optimize the amount of probe and primer for each gene of interest. In monoplex reactions, 100 nM, 200 nM, 300 nM, 400 nM or 500 nM of primers were combined with 200 nM of the corresponding probe and tested using semen cDNA. Once the ideal primer concentrations were determined, the amount of probe in the reaction was optimized using 100 nM, 200 nM and 300 nM.

Once these experiments were carried out for all three candidates, the optimized amounts of primers and probes were combined into a single multiplex reaction and compared to singleplex reactions. The data in Table 23 shows that while each primer/probe set amplified from semen when alone in the reaction, the combination into a multiplex reaction caused the dropout of

TGM4 and B2M amplification. Alternatively, the amplification using the PRM2 primer/probe set was unchanged regardless of the reaction conditions. Future optimization of this assay is required. Since PRM2 is highly expressed in seminal fluid samples, it may be competing against the other sets for reaction components. Changing the primer and probe conditions may address this problem. Furthermore, changing the master mix used in this reaction may have an impact on the results, which has been observed in other studies in our lab.

A second TaqMan®-based multiplex assay which we sought to develop was for the identification of brain tissue. Based on our preliminary studies using the Gene Expression Assays from Applied Biosystems, we identified the genes ADCY1 and GPM6A as potential tissue-specific candidates. Again using the Beacon Designer program, we designed a set of probes and primers for ADCY1 (FAM), GPM6A (CAL Orange 560; HEX) and the housekeeper B2M (CAL Red 610; ROX) which were ordered from Biosearch Technologies. Preliminary experiments were conducted to optimize the amount of probe and primer for each gene of interest. In monoplex reactions, 100 nM, 200 nM, 300 nM, 400 nM or 500 nM of primers were combined with 200 nM of the corresponding probe and tested using cDNA generated from control brain RNA (Ambion). Once the ideal primer concentrations were determined, the amount of probe in the reaction was optimized using 100 nM, 200 nM and 300 nM.

Once these experiments were carried out for all three candidates, the optimized amounts of primers and probes were combined into a single multiplex reaction and compared to singleplex reactions. The data in Table 24 shows that each primer/probe set amplified from brain both when alone in the reaction, and in combination with the other primer/probe sets. Furthermore, there was no significant decrease in the degree of amplification when the sets were alone or

combined into the multiplex. There was some minor amplification in the no template control sample when the ADCY1 primers/probe were alone, however this was not observed when the primers and probes were combined in the multiplex. There was also amplification in the no template controls when the GPM6A set was used. Since the relative expression of this marker is greater than the other two genes, the potential exists to decrease the amount of primers and probe used in the reaction which may alleviate some of this amplification. Also, there was a Ct in the HEX channel for the semen sample which theoretically only received the ADCY1 (FAM) set; this may be due to accidental addition of the GPM6A primers/probe to the reaction tube. However, this multiplex looks very promising for a brain screening assay. We hope to obtain human brain samples from collaborators which would assist in our evaluation of this multiplex as a viable screening assay.

Luminex Technology

A new technology that may offer great promise to the forensic community is the Bio-Plex™ system which uses the multiplexing technology of Luminex Corp. to enable the simultaneous quantitation of up to 100 analytes. This technology uses polystyrene beads (xMAP® beads) internally dyed with differing ratios of two spectrally distinct fluorophores. Each fluorophore can have any of 10 possible levels of fluorescent intensity, thereby creating a family of 100 spectrally addressed bead sets. Multiplex assays can be created by mixing bead sets to simultaneously test for many analytes in one sample. This valuable technique could be used in routine testing and could assess many samples in an automated fashion.

Another adaptation to the Luminex-bead technology is the QuantiGene® Plex Reagent System offered by Panomics. This system combines branched DNA with the multi-analyte profiling beads. Together they enable simultaneous detection of multiple RNA targets directly from purified RNA. Branched DNA technology is a sandwich nucleic acid hybridization assay that amplifies the reporter signal rather than the sequence. Groups have been able to utilize this technology to measure gene expression from blood (Zheng et al., 2006), formalin-fixed, paraffin-embedded tissues (Yang et al., 2006), and directly from cell lysates and tissue homogenates without the need for RNA purification (Zhang et al., 2005; Flagella et al., 2006).

In order to determine whether this technology would work on routine forensic samples, we ordered a 3-plex assay from Panomics which would detect PRM2, SEMG1 and B2M. These were targets that we had a great deal of experience with in terms of our TaqMan® studies; therefore, we had a great deal of data to compare to the new QuantiGene® Plex assay. However, it should be noted that SEMG1 was chosen because at the time this assay was ordered, we had yet to perform the Plexor® Stain ID and TaqMan® assays which ruled it out as a specific marker for seminal fluid (cross-reacted with blood and vaginal secretions, respectively). In our first attempt at running the assay we used a variety of samples. A range of semen samples from 1-20 µl were extracted using TRIzol® as well as a 20 µl blood stain to serve as a control of nonspecific amplification. Testes RNA from Panomics (1 µg/µl) was used as a positive control. Lastly, 1 and 20 µl semen stains were prepared using a direct lysis method recommended by Panomics for the preparation of blood samples. This involved incubating the stains in a lysis buffer (provided with the kit) with proteinase K at 65˚C for 30 minutes. An 80µl aliquot of this lysate (out of 150 µl total) was used in the reaction. Serial dilutions (1:4) of the control testes RNA were made for use in the reaction. Otherwise, 20 µl of the RNA extracts (out of 30 µl

total) were used in each reaction. The results are outlined in Table 25. The amplification of PRM2 was much higher than SEMG1, not surprising based on our TaqMan® studies. There was no nonspecific amplification of PRM2 or SEMG1 with the blood sample as expected. The most interesting finding was that the samples prepared using the lysis buffer and proteinase K performed as well or better than the pure RNA extracts, especially for SEMG1. Because the lysis preparation requires significantly less time than a full extraction, all future experiments will use this procedure.

A more thorough evaluation of the lysate procedure was performed in a subsequent experiment. Several semen stains ranging from 1 to 20 µl (~6 months old) were used in addition to an aged 5x5 mm cutting (~4.5 years old). A 20 µl blood stain was also used to check for nonspecific amplification. Table 26 shows that PRM2 expression was higher than found for SEMG1 or B2M. Furthermore, the expression was highest in the 5x5 mm cutting aged for over 4 years, demonstrating the stability of RNA over time. Since the expression of PRM2 is so much higher than SEMG1, we plan to design a second QuantiGene® Plex assay which will incorporate other seminal fluid targets, such as TGM4 or PSA.

2. Tables

TISSUE	Assays on Demand (ABI)		
Blood	hemoglobin, beta [HBB] (Hs00747223_g1)	CD3G antigen, gamma polypeptide [CD3G] (Hs00173941_m1)	spectrin, beta, erythrocytic [SPTB] (Hs00165820_m1)
Semen	semenogelin I [SEMG1] (Hs00268141_m1)	kallikrein 3 [PSA] (Hs00426859_g1)	acid phosphatase, prostate [ACPP] (Hs00173475_m1)
	cysteine-rich secretory protein 1[CRISP1] (Hs00538261_m1)	microseminoprotein, beta- [MSMB] (Hs00159303_m1)	transglutaminase 4 [TGM4] (Hs00162710_m1)
Sperm	protamine 2 [PRM2] (Hs00172518_m1)		
Saliva	statherin [STATH] (Hs00162389_m1)	histatin 3 [HTN3] (Hs00264790_m1)	
Urine	renin [REN] (Hs00166915_m1)	uroplakin 2 [UPK2] (Hs00171854_m1)	
Brain	dopamine receptor 1 [DRD1] (Hs00377719_g1)	glycoprotein M6A [GPM6A] (Hs00245530_m1)	adenylate cyclase 1, brain [ADCY1] (Hs00299832_m1)
Liver	phospho-fructokinase, liver [PFKL] (Hs00160027_m1)	lipase, hepatic [LIPC] (Hs00165106_m1)	transcription factor 2, hepatic [TCF2] (Hs00172123_m1)
	carnitine palmitoyltransferase 1A [CPT1A] (Hs00157079_m1)	phosphorylase glycogen [PYGL] (Hs00161132_m1)	liver expressed antimicrobial peptide 2 [LEAP2] (Hs00364834_m1)
	glycogen synthase 2 [GYS2] (Hs00608677_m1)		
Heart	troponin T Type 2 (cardiac) [TNNT2] (Hs00165960_m1)	ryanodine receptor 2 (cardiac) [RYR2] (Hs00181461_m1)	myosin binding protein C, cardiac [MYBPC3] (Hs00165232_m1)
Intestine	annexin A13 [ANXA13] (Hs00188802_m1)	alkaline phosphatase intestinal [ALPI] (Hs00357578_m1)	fatty acid binding protein 2, intestinal [FABP2] (Hs00164552_m1)
Kidney	aquaporin 6, kidney specific [AQP6] (Hs00364989_m1)	cadherin 16, KSP-cadherin [CDH16] (Hs00184865_m1)	chloride channel Ka [CLCNKA] (Hs00427895_g1)
	dicarbonyl/L-xylulose reductase [DCXR] (Hs00212433_m1)	myo-inositol oxygenase [MIOX] (Hs00367743_m1)	ankyrin repeat domain 15 [ANKRD15] (Hs00299498_m1)
Control	beta 2 microglobulin [B2M] (Hs99999907_m1)		

Table 1a. Assays-on-Demand™ Gene Expression products used in the grant.

		ASSAY						
Candidate	**Theoretical Target**	**ABI TaqMan® (monoplex)**	**In-House TaqMan® (Semen-Sperm)**	**In-House TaqMan® (Brain)**	**In-House Plexor® (Blood-Semen)**	**In-House Plexor® (Semen-Sperm)**	**Plexor Stain ID**	**QuantiGene® (Semen-Sperm)**
ACPP	Semen	screened				final assay		
ADCY1	Brain	screened		final assay				
ALPI	Intestine	screened						
ANKRD15	Kidney	screened						
ANXA13	Intestine	screened						
AQP6	Kidney	screened						
B2M	Housekeeper	screened	final assay	final assay		screened		screened
CD3G	Blood	screened			screened			
CDH16	Kidney	screened						
CLCNKA	Kidney	screened						
CPT1A	Liver	screened						
CRISP1	Semen	screened						
DCXR	Kidney	screened						
DRD1	Brain	screened						
GAPDH	Housekeeper						final assay	
GAPDHS	Sperm					final assay		
GPM6A	Brain	screened		final assay				
GYS2	Liver	screened						
HBB	Blood	screened			final assay			
HTN3	Saliva	screened					final assay	
LEAP2	Liver	screened						
LIPC	Liver	screened						
MIOX	Kidney	screened						
MSMB	Semen	screened					screened	
MYBPC3	Heart	screened						
PFKL	Liver	screened						
PRM2	Sperm	screened	final assay		final assay	final assay	screened	screened
PSA	Semen	screened			final assay	final assay	screened	
PYGL	Liver	screened						
REN	Urine	screened						
RYR2	Heart	screened						
SEMG1	Semen	screened				screened	screened	screened
SPTB	Blood	screened			screened			
STAT	Saliva	screened						
TCF2	Liver	screened						
TGM4	Semen	screened	final assay				final assay	
TNNT2	Heart	screened						
UPK2	Urine	screened						

Table 1b. Use of gene candidates throughout the grant.

SAMPLE	**B2M (Cts)**
NTC	ND
Kidney #1	25.11
Kidney #2	23.36
Kidney #3	24.79
Kidney #4	18.77
Adipose #1	18.69
Adipose #2	24.44
Colon #1	26.64
Colon #2	20.59
Skin #1	21.18

Table 2. mRNA from human tissues amplified with B2M TaqMan® assay.

Sample	Organic	BioRobot® M48	Sucrose
Blood (n = 12)			
AVG ± SD	2.75 ± 1.54 ng/µl	2.50 ± 5.99 ng/µl	2.93 ± 0.98 ng/µl
Range	0.15 - 4.98 ng/µl	0.20 - 21.4 ng/µl	0.75 - 4.80 ng/µl
Buccal (n = 12)			
AVG ± SD	0.99 ± 1.21 ng/µl	3.68 ± 3.12 ng/µl	2.76 ± 2.89 ng/µl
Range	0.07 - 3.19 ng/µl	0.48 - 9.99 ng/µl	0.30 - 8.54 ng/µl

Table 3. Comparison of DNA yields from Organic, BioRobot® M48 and Sucrose Methods.

	Organic		BioRobot® M48		Sucrose	
Sample	*D3S1358* Peak Height (RFUs)	*CSF1PO* Peak Height (RFUs)	*D3S1358* Peak Height (RFUs)	*CSF1PO* Peak Height (RFUs)	*D3S1358* Peak Height (RFUs)	*CSF1PO* Peak Height (RFUs)
Blood						
Databank #1	670/772	565/707	1366/1545	1383/1596	562/637	459/496
Databank #2	646/732	929	1049/1307	2040	895/871	1261
Databank #3	524/656	855	1178/1195	1866	769/779	1156
Databank #4	535/604	312/344	939/1068	959/977	542/652	462/495
Databank #5	402/528	338/350	1274/1283	1094/1392	648/774	481/513
Databank #6	1119	1445	1636	1319	1127	1157
Databank #7	643/703	712/754	953/1051	908/1031	1073/1288	980/1006
Databank #8	486/633	490/615	933/972	968/1008	810/851	561/664
Databank #9	507/604	772	781/851	1648	554/634	931
Databank #10	586/688	284/225	796/1002	728/834	910/914	785/786
Databank #11	1018/1175	602/628	682/774	741/832	826/832	618/643
Databank #12	805/932	379/584	944/985	916/1097	807/877	388/523
Buccal						
Databank #1	562/672	574/590	380/385	226/283	1215/1366	487/518
Databank #2	999/1316	2533	212/257	394	1078/1226	1799
Databank #3	611/701	1049	553/1020*	1080	1185/1431	606
Databank #4	385/550	402/451	357/407	177/254	999/1007	451/520
Databank #5	623/657	310/506	672/693	557/802	658/759	405/431
Databank #6	462/676	291/338	995/1258	602/931	853/865	363/368
Databank #7	1228	671/676	1353	479/777	1491	560/563
Databank #8	421/506	782	677/764	1645	1154/1205	1254
Databank #9	1206	659	1098	743	1179	358
Databank #10	759/834	743/763	430/475	544/603	1469/1702	1201/1253
Databank #11	854/1013	2083	646/766	1393	712/822	1179
Databank #12	880/1023	695/752	355/429	298/352	687/881	714/761

*Heterozygous peak ratio < 60%

Table 4. STR analysis results with the Identifiler™ Kit.

BLOOD	AGE (DAYS)	HBB Ct
1 µl	3	19.94
1 µl	23	19.99
1 µl	461	23.30
1 µl	496	23.52
1 µl	510	23.15
10 µl	3	17.68
10 µl	420	19.94
10 µl	490	20.27
10 µl	855	20.15
10 µl	864	20.51

Ct for the NTC was 35.15

Table 5. Time course of HBB expression in blood stains.

SEMEN	AGE (DAYS)	PRM2 Ct
1 µl	16	34.61
1 µl	461	30.35
1 µl	507	27.41
1 µl	699	30.95
1 µl	807	36.90
20 µl	16	27.92
20 µl	507	20.10
20 µl	607	22.19
20 µl	777	21.34
20 µl	791	22.94

Ct for NTC was not observed during the 50 cycles

Table 6. Time course of PRM2 expression in seminal fluid stains.

BLOOD VOLUME	HBB	CD3G	SPTB
NTC	37.21	ND	ND
0.0001 µl	33.13	ND	ND
0.001 µl	29.92	ND	ND
0.01 µl	26.28	ND	ND
0.1 µl	24.31	ND	ND
1 µl	21.60	33.41	35.00
5 µl	19.08	29.63	34.35
10 µl	16.67	28.69	31.56
20 µl	15.84	28.62	31.24

ND = not detected

Table 7. Sensitivities of TaqMan® blood assays.

SEMEN VOLUME	SEMG1	PSA	MSMB	TGM4	PRM2
NTC	ND	ND	ND	ND	ND
0.01 µl	ND	37.20	N/A	N/A	35.67
0.1 µl	33.79	34.15	N/A	N/A	32.26
1 µl	30.80	31.39	25.42	28.33	28.93
5 µl	31.43	32.48	28.90	30.69	27.57
10 µl	31.55	35.13	29.74	31.15	24.80
20 µl	31.86	34.02	28.35	30.79	27.24

ND = not detected

N/A = not determined

Table 8. Sensitivities of TaqMan® semen and sperm assays.

[TESTES RNA]	SEMG1	MSMB	TGM4	PRM2
NTC	ND	ND	ND	ND
1 ng	ND	ND	35.93	25.29
10 ng	ND	33.88	36.18	20.89
100 ng	35.2	29.42	31.67	17.39
1 µg	33.39	26.49	29.67	16.06

ND = not detected

Table 9. Sensitivities of TaqMan® semen and sperm assays using control RNA.

SALIVA VOLUME	STAT	HTN3
NTC	ND	ND
0.1 µl	ND	ND
1 µl	34.14	ND
5 µl	33.93	39.45
10 µl	37.80	37.93
20 µl	34.68	36.25

ND = not detected

Table 10. Sensitivities of TaqMan® saliva assays.

	BLOOD			SEMEN						SPERM	SALIVA	CONTROL
	HBB	SPTB	CD3G	PSA	SEMG1	ACPP	CRISP1	MSMB	TGM4	PRM2	STAT	B2M
Blood	+	+	+	-	-	+	-	-	-	-	-	+
Semen	-	-	-	+	+	+	-	+	+	+	-	+
Saliva	-	-	-	-	-	-	-	-	-	-	+	+
Menstrual Blood					-	-	-	-	-	-		+
Vaginal Secretions					+	+	-	-	-	-		+
Kidney	-			-	-			-	-	-		+
Colon					-			-	-	-		+
Adipose					-			-	-	-		+
Skin					-			-	-	-		+
Ambion Brain					-			-	-	-		+
Ambion Heart					-			-	-	-		+
Ambion Liver					-			-	-	-		+
Ambion Kidney					-			-	-	-		+
Ambion Intestine					-			+	-	-		+

Table 11. Specificity of blood, semen, sperm and saliva TaqMan® assays (grey = not tested).

	BRAIN			HEART			INTESTINE		
	DRD1	**GPM6A**	**ADCY1**	**RYR2**	**MYBPC3**	**TNNT2**	**ANXA13**	**ALPI**	**FABP2**
Blood	-	-	-	-	-	-	-	-	-
Semen	-	-	-	-	-	-	-	-	-
Saliva	-	-	-	-	-	-	-	-	-
Menstrual Blood							-	-	-
Vaginal Secretions							-	-	-
Kidney							+	+	+
Colon							+	+	+
Adipose							-	-	-
Skin							-	-	-
Ambion Brain	+	+	+	-	-	-	-	-	-
Ambion Heart	-	-	+	+	+	+	-	-	-
Ambion Liver	+	-	-	-	-	-	-	-	-
Ambion Kidney	-	-	+	-	-	-	-	-	-
Ambion Intestine	+	-	-	-	-	-	+	+	+

Table 12. Specificity of brain, heart and intestine TaqMan® assays (grey = not tested).

	LIVER							KIDNEY					
	PFKL	**LIPC**	**TCF2**	**CPT1A**	**PYGL**	**LEAP2**	**GYS2**	**AQP6**	**CDH16**	**CLCNKA**	**DCXR**	**MIOX**	**ANKRD15**
Blood	-	-	-	-	+	-	-	-	-	-	-	-	-
Semen	-	-	-	-	-	-	-	-	-	-	-	-	-
Saliva	-	-	-	-	-	-	-	-	-	-	-	-	-
Menstrual Blood													
Vaginal Secretions													
Kidney								-	+	+		+	+
Colon								-	+	+		-	
Adipose								-	-	+		-	
Skin								-	-	+		-	
Ambion Brain	+	-	-	+	-	-	-	-	+	-	+	-	+
Ambion Heart	+	-	-	+	+	-	-	-	+	-	+	-	+
Ambion Liver	+	+	+	+	+	+	+	-	+	-	+	-	+
Ambion Kidney	+	+	+	+	+	+	-	-	+	+	+	+	+
Ambion Intestine	+	-	+	+	+	+	-	-	+	-	+	-	+

Table 13. Specificity of liver and kidney TaqMan® assays (grey = not tested).

SAMPLE	HBB (FAM)	PSA (HEX)	PRM2 (ROX)
NTC	ND	ND	31.4
Blood (No RT)	ND	ND	30.2
Semen (No RT)	30.3	32.0	32.0
1:1 Mixture (No RT)	31.5	ND	33.2
Blood	**18.5**	ND	30.6
Semen	29.7	**25.3**	**21.4**
1:1 Mixture	**20.3**	**26.1**	**21.5**

ND = Not Detected

Table 14. Blood and semen detection by a Plexor®-based multiplex assay.

SAMPLE	GAPDHS (FAM)	ACPP (HEX)	PRM2 (ROX)
NTC	31.7	30.9	31.6
Blood (No RT)	31.8	ND	ND
Semen (No RT)	27.3	ND	31.9
1:1 Mixture (No RT)	28.9	ND	ND
Blood	30.1	28.3	31.4
Semen	**21.3**	**24.1**	**21.4**
1:1 Mixture	**21.9**	**24.5**	**22.3**

ND = Not Detected

Table 15. Semen detection by a Plexor®-based seminal fluid multiplex assay.

SAMPLE	PSA (CY5)
NTC	36.9
No DNase Treatment (No RT)	33.8
DNase Treatment (No RT)	37.3
No DNase Treatment	28.8
DNase Treatment	30.7

Table 16. Semen detection by Plexor® PSA primers of samples with and without DNase treatment.

SAMPLE	GAPDHS (FAM)	ACPP (HEX)	PRM2 (ROX)	PSA (CY5)
NTC	28.9	31.8	22.7	29.6
Smen (No RT)	35.2	ND	29.9	32.7
Semen	27.2	25.5	7.6	25.7

ND = Not Detected

Table 17. Semen detection by a Plexor®-based seminal fluid multiplex assay.

SAMPLE	HTN3 (FAM)	SEMG1 (HEX)	GAPDH (ROX)
NTC	ND	ND	ND
NTC	ND	ND	ND
DNA	ND	34.3	ND
DNA	ND	33.5	ND
vaginal secretion	ND	ND	22.1
vaginal secretion	ND	ND	22.3
vagina	ND	ND	21.4
vagina	ND	ND	21.1
submaxillary gland	**8.8**	ND	24.7
submaxillary gland	**8.2**	ND	24.8
salivary gland	**14.8**	ND	26.0
salivary gland	**14.8**	ND	25.8
saliva	**27.0**	ND	28.0
saliva	**26.9**	ND	27.2
skin	ND	ND	23.0
skin	ND	ND	23.0
semen	ND	**19.7**	25.1
semen	ND	**19.8**	25.5
blood	ND	ND	24.9
blood	ND	ND	25.5

ND = Not Detected

Table 18. Detection of semen and saliva using a Plexor®-based Stain ID assay.

SAMPLE	HTN3 (FAM)	TGM4 (HEX)	GAPDH (ROX)
NTC	ND	ND	ND
Blood (20ul)	ND	ND	30.1
Semen (20ul)	ND	**27.5**	34.9
Semen (10ul)	ND	**27.0**	35.9
Semen (5ul)	ND	**25.6**	33.5
Semen (1ul)	ND	**25.0**	35.3
Saliva (20ul)	**20.9**	ND	ND
Saliva (10ul)	**22.3**	ND	ND
Saliva (5ul)	**22.6**	ND	ND
Saliva (1ul)	**25.4**	ND	ND
Menstrual Blood	ND	ND	38.8
Vaginal Secretions	24.4	ND	24.4
Semen (+) Control	ND	**24.4**	36.5
Saliva (+) Control	**21.6**	ND	37.5

ND = Not Detected

Table 19. Detection of semen and saliva with the Plexor® Stain ID assay.

	Pre-Processing Step		No Pre-Processing Step	
SAMPLE	**Tris (ng)**	**RNagents (ng)**	**Tris (ng)**	**RNagents (ng)**
10ul semen	2851.25	21.07	361.88	0.53
1ul semen	190.78	3.65	63.58	0.27
10ul saliva	166.45	0.56	89.38	0.94
1ul saliva	3.49	0.01	6.66	0.04

Table 20. DNA yield comparison of Tris-buffered phenol and RNagents extractions.

	Pre-Processing Step		No Pre-Processing Step	
SAMPLE	**Tris (Ct)**	**RNagents (Ct)**	**Tris (Ct)**	**RNagents (Ct)**
10ul semen	35.1	39.2	22.9	22.1
1ul semen	38.1	37.6	25.2	24.8
10ul saliva	ND	ND	29.6	30.3
1ul saliva	ND	ND	34.6	34.8

ND = not detected

Table 21. RNA yield comparison of Tris-buffered phenol and RNagents extractions.

SAMPLE	**DNA (ng/ul)**	**HTN3 RNA (Ct)**	**TGM4 RNA (Ct)**	**GAPDH RNA (Ct)**
10ul semen	17.3	ND	21.9	23.7
1ul semen	2.0	ND	25.0	25.0
10ul saliva	13.8	29.0	ND	29.0
1ul saliva	1.3	32.9	ND	32.4

Table 22. DNA yields and RNA expression of semen and saliva samples extracted with the Tris-buffered phenol procedure.

SAMPLE (PRIMER/PROBE SET USED)	**FAM Channel (PRM2)**	**HEX channel (TGM4)**	**ROX channel (B2M)**
NTC (PRM2)	ND	ND	ND
Semen (PRM2)	19.84	ND	ND
NTC (TGM4)	ND	ND	ND
Semen (TGM4)	ND	26.19	ND
NTC (B2M)	ND	ND	ND
Semen (B2M)	ND	ND	34.59
NTC (Multiplex)	ND	ND	ND
Semen (Multiplex)	19.74	ND	ND

ND = Not Detected

Table 23. Detection of semen using a multiplex TaqMan® assay.

SAMPLE (PRIMER/PROBE SET USED)	FAM Channel (ADCY1)	HEX channel (GPM6A)	ROX channel (B2M)
NTC (ADCY1)	36.34	ND	ND
Brain (ADCY1)	20.11	23.53	ND
NTC (GPM6A)	ND	20.46	ND
Brain (GPM6A)	ND	15.64	ND
NTC (B2M)	ND	ND	ND
Brain (B2M)	ND	ND	19.45
NTC (Multiplex)	ND	20.57	ND
Brain (Multiplex)	20.27	15.58	19.97

ND = Not Detected

Table 24. Detection of brain using a multiplex TaqMan® assay.

SAMPLE	PRM2 (fold change)	SEMG1 (fold change)	B2M (fold change)
testes RNA (100ng/ul)	1140.8	1.3	2235.9
testes RNA (25ng/ul)	461.8	0.8	908.2
testes RNA (6.25ng/ul)	175.1	1.0	266.3
testes RNA (1.56ng/ul)	48.2	0.9	59.0
semen (20ul)	36.2	2.3	2.4
semen (10ul)	19.4	2.4	3.5
semen (5ul)	18.9	4.5	2.1
semen (1ul)	5.5	2.5	2.2
blood (20ul)	0.8	0.8	555.8
semen lysate (20ul)	39.2	9.6	2.8
semen lysate (1ul)	6.3	5.1	3.5

Table 25. Expression of PRM2, SEMG1 and B2M in semen and blood samples using a QuantiGene® Plex assay.

SAMPLE	PRM2 (fold change)	SEMG1 (fold change)	B2M (fold change)
semen lysate (20ul)	13.5	3.7	3.2
semen lysate (10ul)	6.9	3.6	2.9
semen lysate (5ul)	9.1	3.3	3.5
semen lysate (1ul)	2.4	2.7	2.5
semen lysate (5x5mm)	660.5	9.7	2.2
blood lysate (20ul)	0.7	1.3	422.0

Table 26. Expression of PRM2, SEMG1 and B2M in semen and blood lysates using a QuantiGene® Plex assay.

METHOD	TIME/SAMPLE	COST/SAMPLE
Phenol:Chloroform	~2 hr + 8-18 hr incubation	$3.58
M48 BioRobot®	~1 hr + 1-4 hr incubation	$5.72
Sucrose	90 min	$1.76

Table 27. Time and cost analysis of DNA extraction methods.

3. Figures

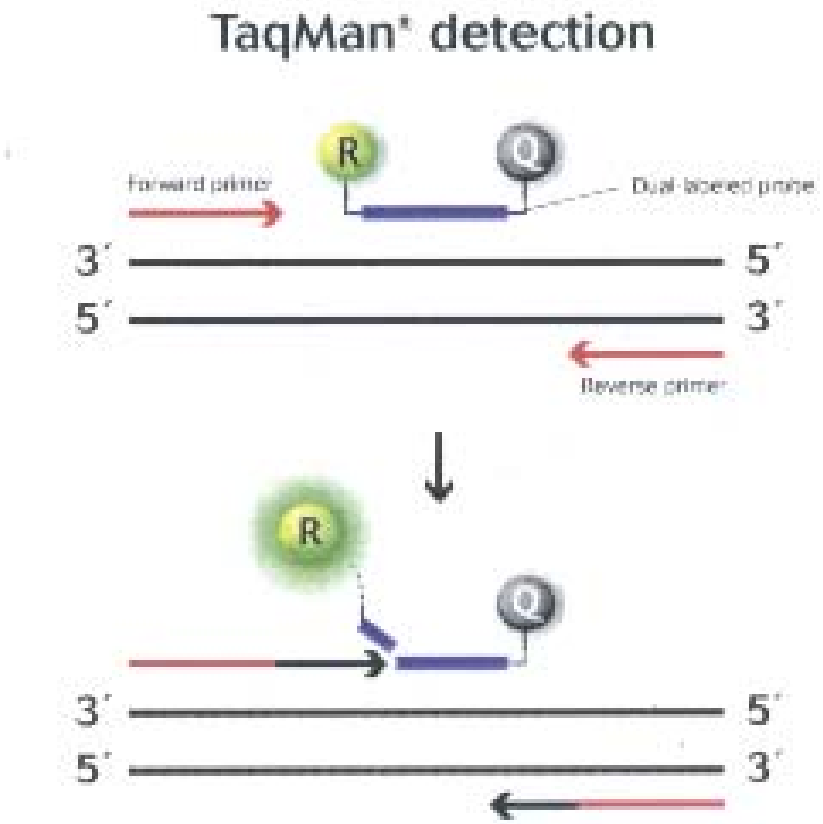

Figure 1. Schematic of TaqMan® based real-time PCR analysis. Each reaction contains a gene specific primer and a fluorescently labeled TaqMan® probe. The probe contains a 5' reporter dye and a 3' quencher dye. The probe is designed to anneal to the target sequence between the forward and reverse PCR primers. While the probe is intact, the quencher suppresses the fluorescence of the reporter dye. During amplification, Taq DNA polymerase cleaves the probe and displaces it from the target, allowing extension to continue. Cleavage of the probe separates the reporter dye from the quencher dye, resulting in an increase in fluorescence. The increased fluorescence only occurs if the target sequence is amplified and is complimentary to the probe, thus preventing detection of non-specific amplification.

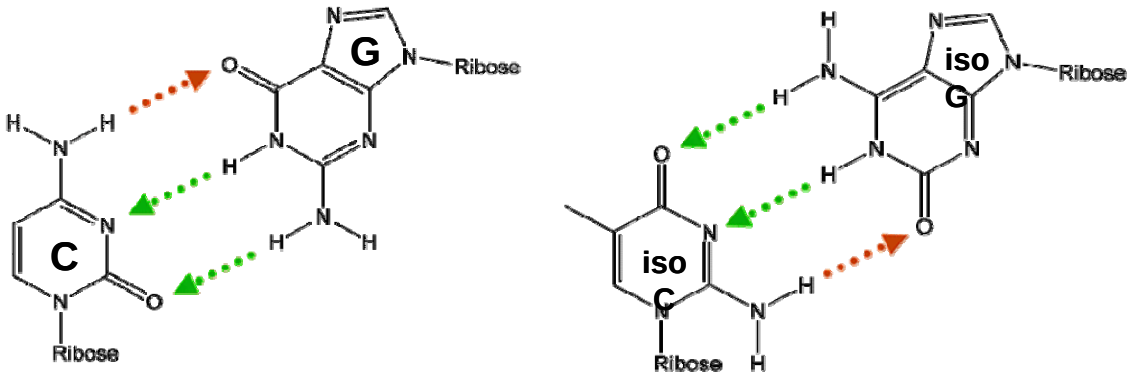

Figure 2. Schematic of synthetic isoC and isoG bases developed by Eragen Biosciences. Eragen Biosciences developed and synthesized a series of new DNA base pairs. The Plexor® technology is based on one of these new base pairings: isoG and isoC. IsoC and IsoG nucleotides are incorporated by DNA Polymerase; however, neither isoC nor isoG can base pair with any of the other conventional bases. These two novel bases only interact and base pair with one another and are not found in nature. Although similar to the conventional G-C pair, you can see the hydrogen bonding pattern is much different.

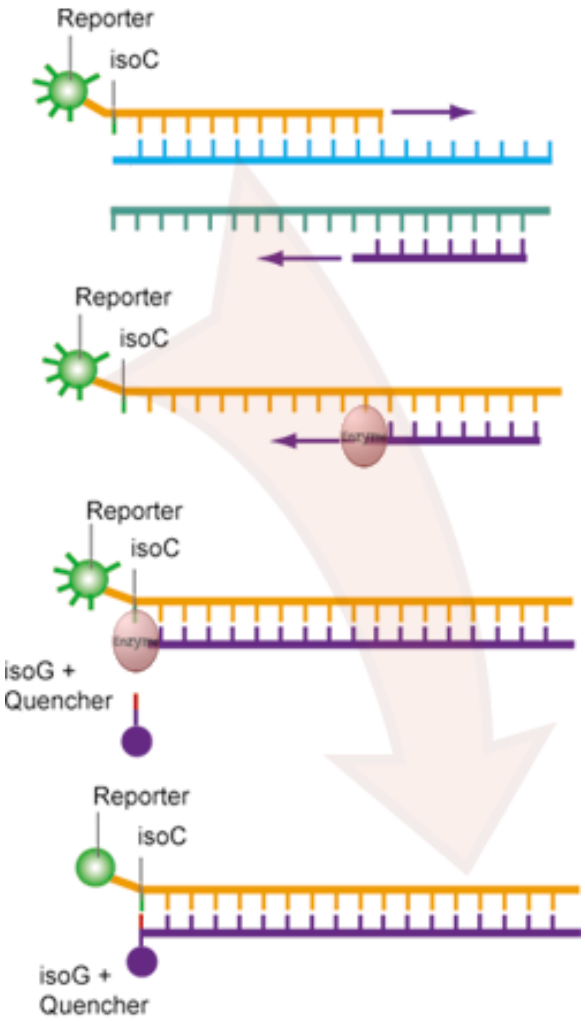

Figure 3. Schematic of Plexor® chemistry. Plexor® takes a conventional PCR primer and adds the isoC base to the 5' end of the primer. To this is attached a fluorescent reporter, by conventional 5' end labeling methods. The Plexor® Primer and a normal downstream PCR primer begin the process of replicating the DNA sequence of interest. This process is fed by conventional dNTPs, as with any amplification. At the end of the amplimer, the polymerase is confronted with the isoC base. The Plexor® Master Mix contains isoG bases that are labeled with the quencher dabcyl. The pairing of the isoC and isoG bases brings the dabcyl quencher within close proximity of the fluor, resulting in very efficient quenching of the fluorescent reporter.

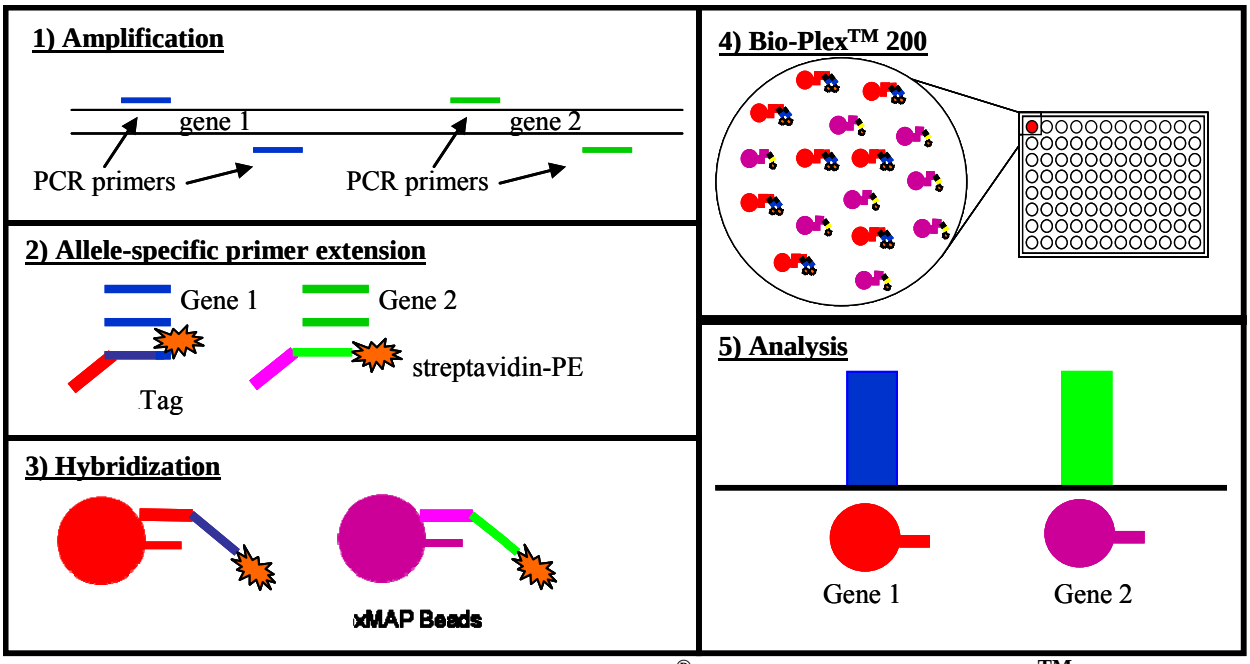

Figure 4. Gene expression analysis using xMap® Beads and the Bio-Plex™ 200. PCR is performed using two primers flanking each gene of interest. As shown, two probes are present in the second reaction: each one contains a distinct FlexTag attached to a sequence complementary to the amplified gene of interest. In the allele-specific primer extension, a DNA polymerase is used for primer extension and biotin-dCTP label incorporation. The tagged, biotin-labeled products are captured by their tag complements (anti-tags) on FlexMap microspheres by hybridization. Streptavidin-phycoerythrin is used to indirectly detect the ASPE-incorporated biotin. Following analysis on the BioPlex™ 200, the signals generated are used to detect the presence or absence of each gene of interest within the sample.

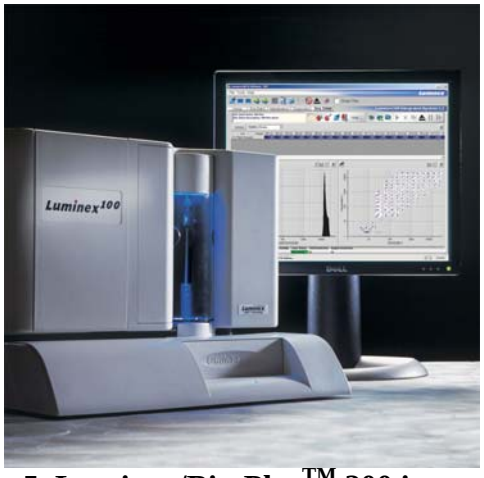

Figure 5. Luminex/Bio-Plex™ 200 instrument.

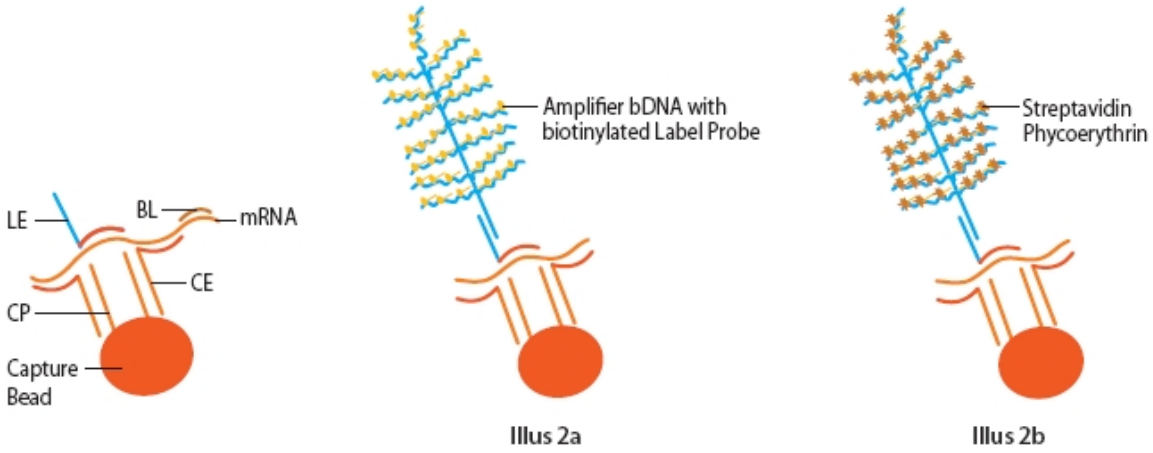

Figure 6. Schematic of branched DNA signal amplification and xMAP® bead technologies. Specific mRNA transcripts are captured to their respective beads through Capture Extender (CE) Capture Probe (CP) interaction during an overnight hybridization at 53°C. Sequential hybridization of the bDNA amplification molecule and biotinylated Label Probe, respectively, for an hour at 46°C. Binding with Streptavidin-conjugated Phycoerythrin (SA-PE) at room temperature for 30 minutes. The sample is analyzed on a Luminex instrument. The level of SA-PE fluorescence is proportional to the amount of mRNA transcripts captured by the bead.

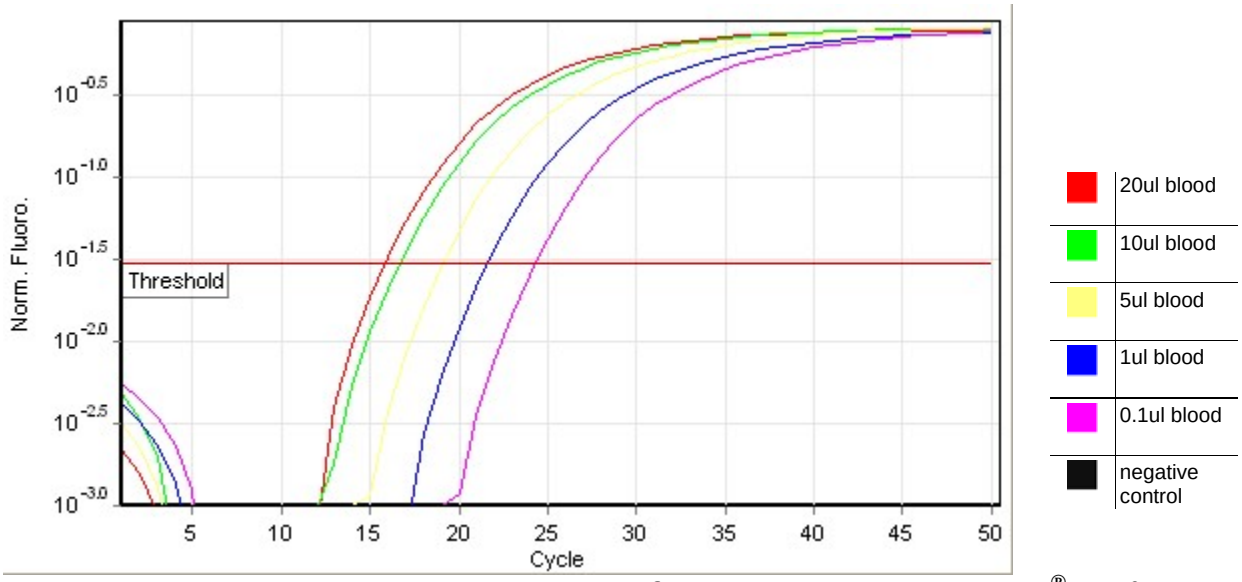

Figure 7. HBB expression in RNA extracted from dried blood by TRIzol®. After room temperature storage for 18 hours, dried blood stains (0.01 µl – 20 µl) were extracted using the TRIzol® reagent. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the HBB Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

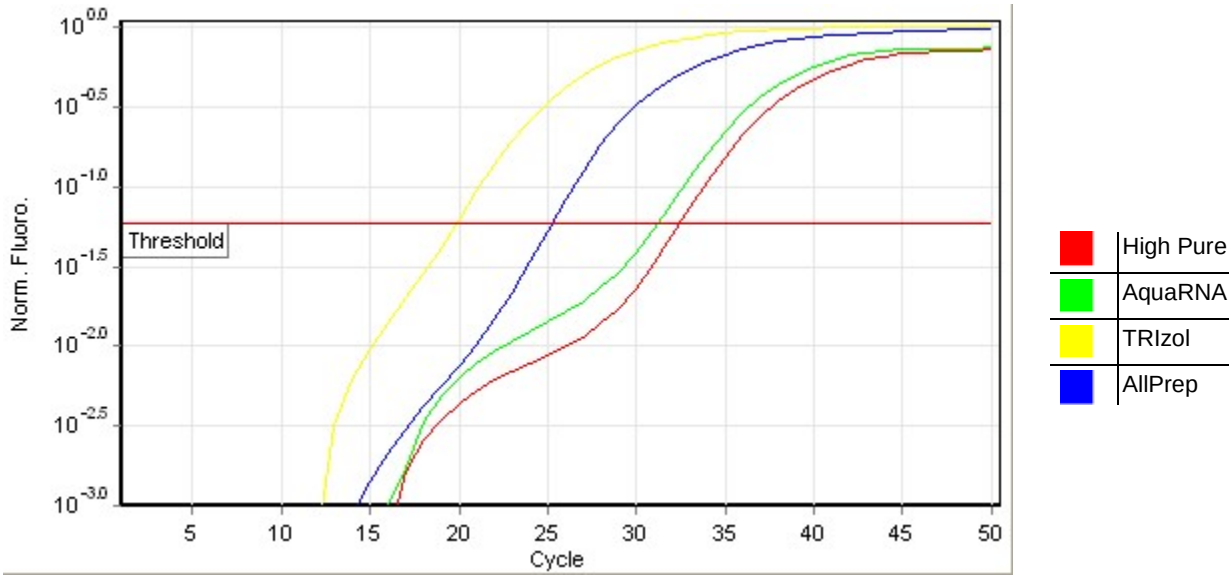

Figure 8. Comparison of HBB expression in RNA extracted from 1 µl of dried blood. After room temperature storage for 771 days, dried blood stains (1 µl) were extracted using the High Pure RNA Isolation Kit, AquaRNA Kit, TRIzol® reagent and AllPrep DNA/RNA Mini Kit. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the HBB Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

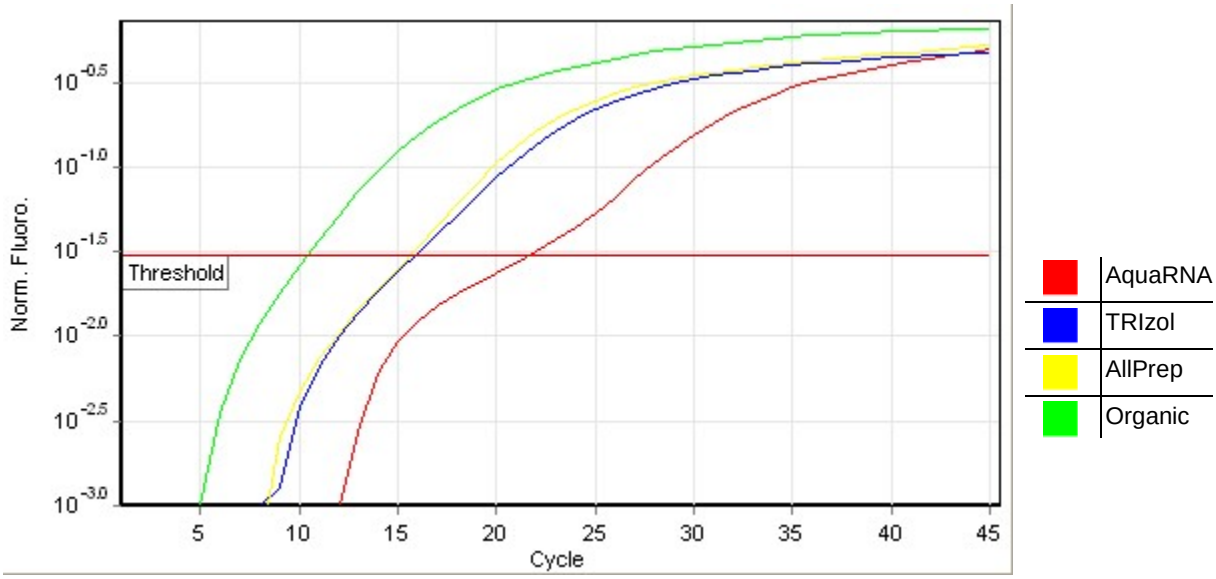

Figure 9. Total DNA yields in DNA extracted from 20 µl of dried blood by four extraction methods. After room temperature storage for 127 days, dried blood stains (20 µl) were extracted using the AquaRNA Kit, TRIzol® reagent, AllPrep DNA/RNA Mini Kit and Organic method. Two µl of DNA was quantitated using the TaqMan® duplex human/Y DNA quantitation assay. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

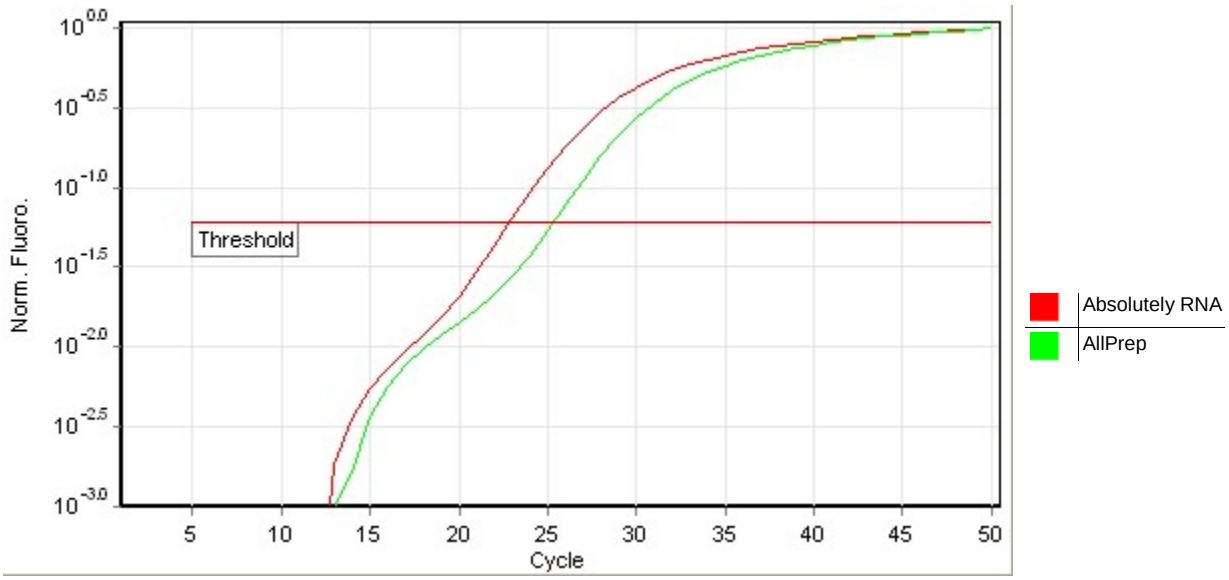

Figure 10. PRM2 expression in RNA extracted from 10 µl of dried seminal fluid by two extraction methods. After room temperature storage for 288 days, dried semen stains (10 µl) were extracted using the Absolutely RNA® Kit and AllPrep DNA/RNA Mini Kit. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the PRM2 Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

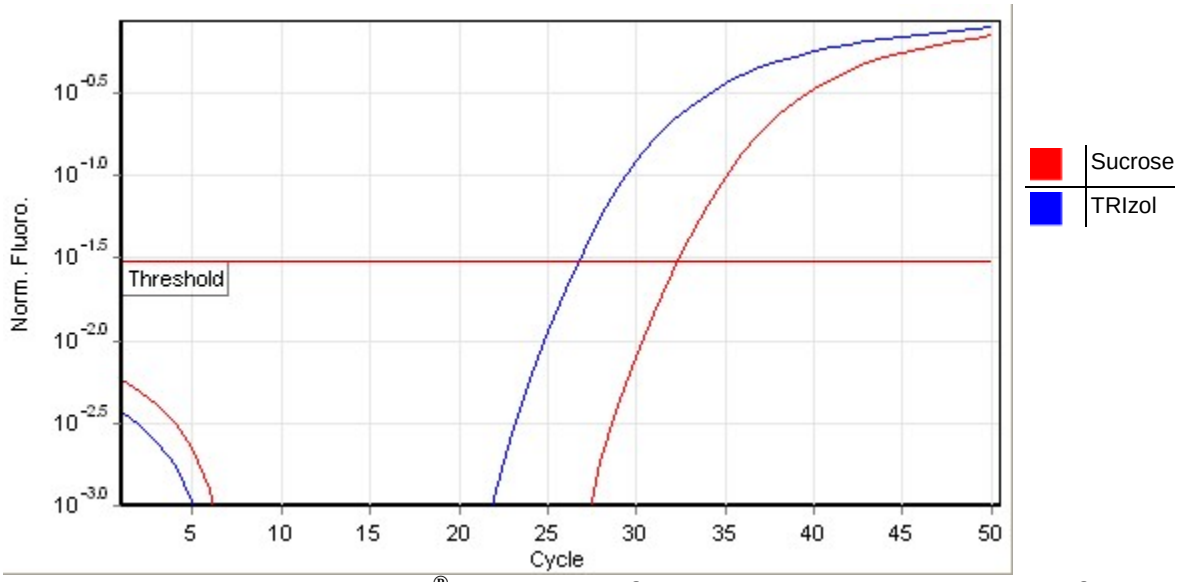

Figure 11. Sucrose vs. TRIzol® extraction of 1ul dried blood stains – amplification with HBB TaqMan® assay. After room temperature storage for 2 days, dried blood stains (1 µl) were extracted using the TRIzol® reagent and Sucrose Method. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the HBB Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

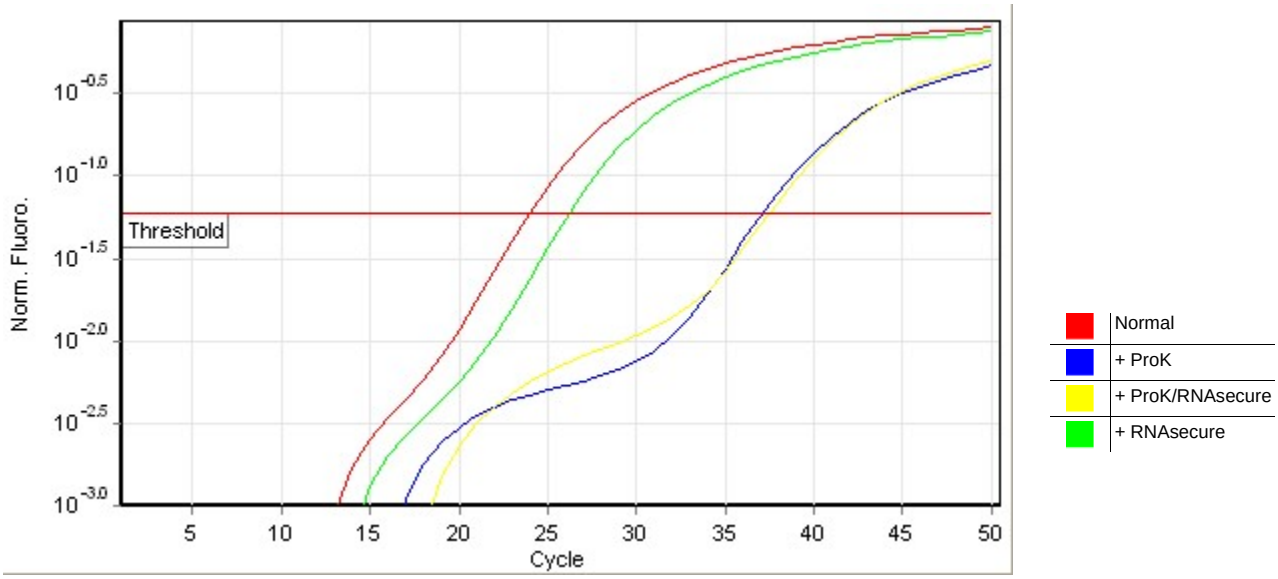

Figure 12. Troubleshooting the Sucrose Method to enhance RNA extraction. After room temperature storage for 3 days, dried blood stains (20 µl) were extracted using the Sucrose Method with various adaptations: 1) normal procedure, 2) proteinase K added to sucrose solution and heated at 56˚C for 1 hr prior to the normal 100˚C incubation, 3) RNA*secure*™ added to sucrose solution, incubated at 60˚C for 10 minutes, proteinase K added to sucrose solution as previously described, and 4) RNA*secure*™ added to sucrose solution as previously described. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the HBB Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

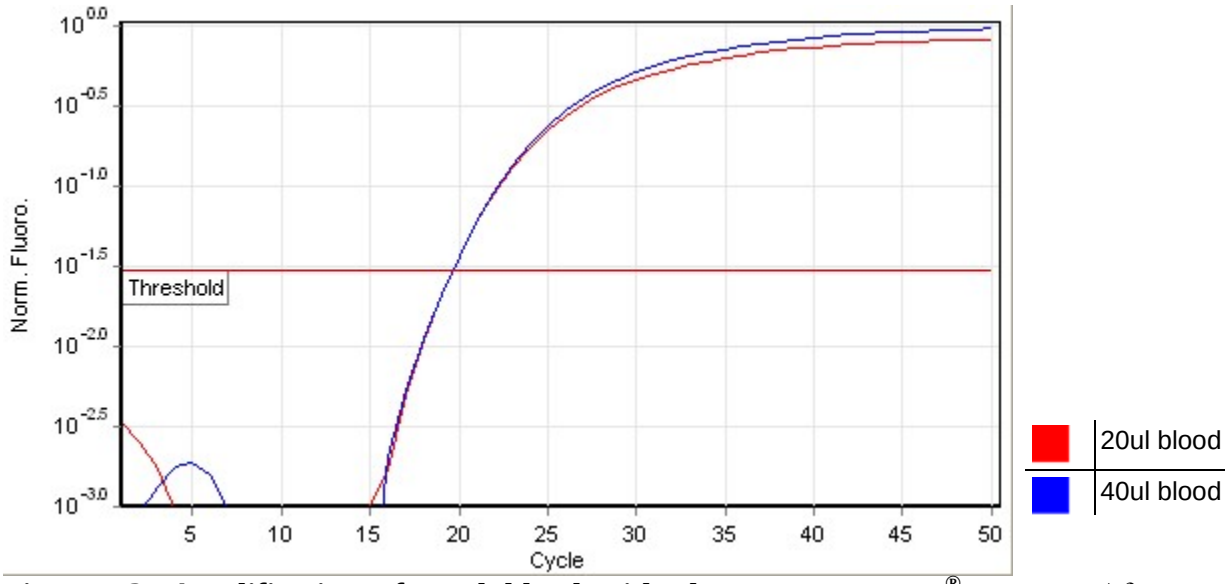

Figure 13. Amplification of aged blood with the HBB TaqMan® assay. After room temperature storage for 13 or 26 months, dried blood stains (20 and 40 µl, respectively) were extracted using the TRIzol® reagent. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the HBB Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

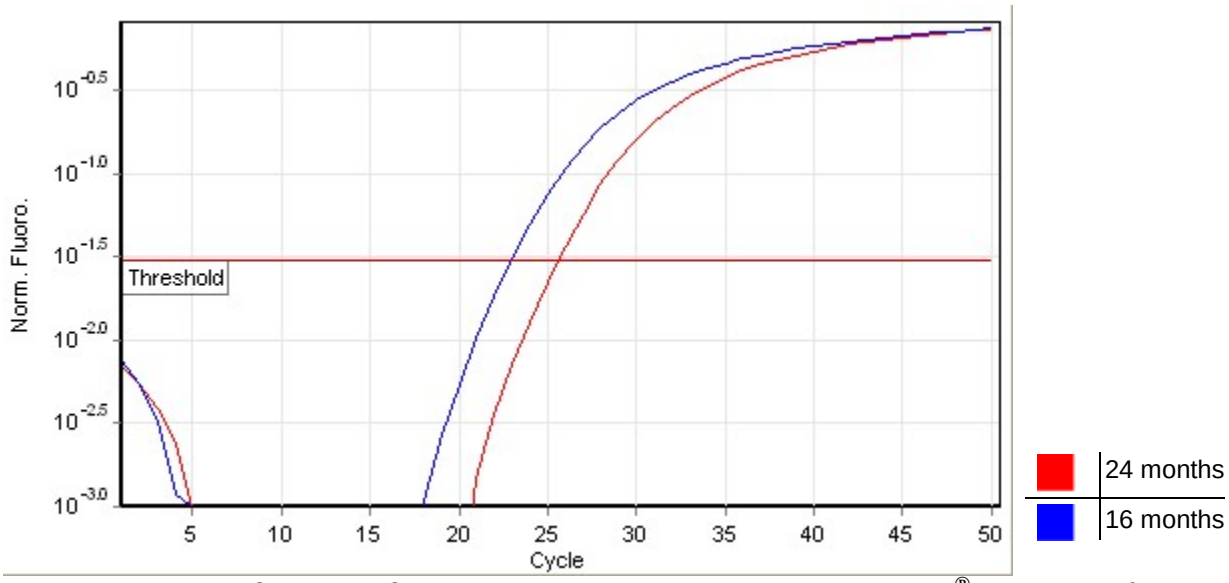

Figure 14. Amplification of aged semen with the PRM2 TaqMan® assay. After room temperature storage for 16 or 24 months, dried semen stains (20 µl) were extracted using the TRIzol® reagent. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the PRM2 Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

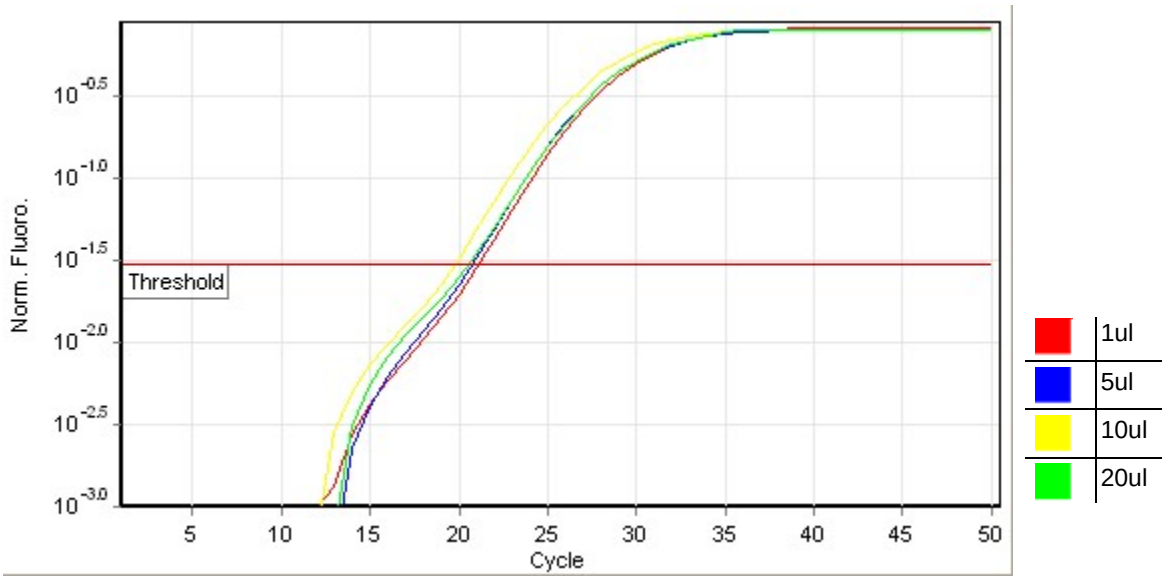

Figure 15. Expression of HBB in aged blood samples extracted by the AllPrep Kit. After room temperature storage for 752 days, dried blood stains (1-20 µl) were extracted using the AllPrep DNA/RNA Mini Kit. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the HBB Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

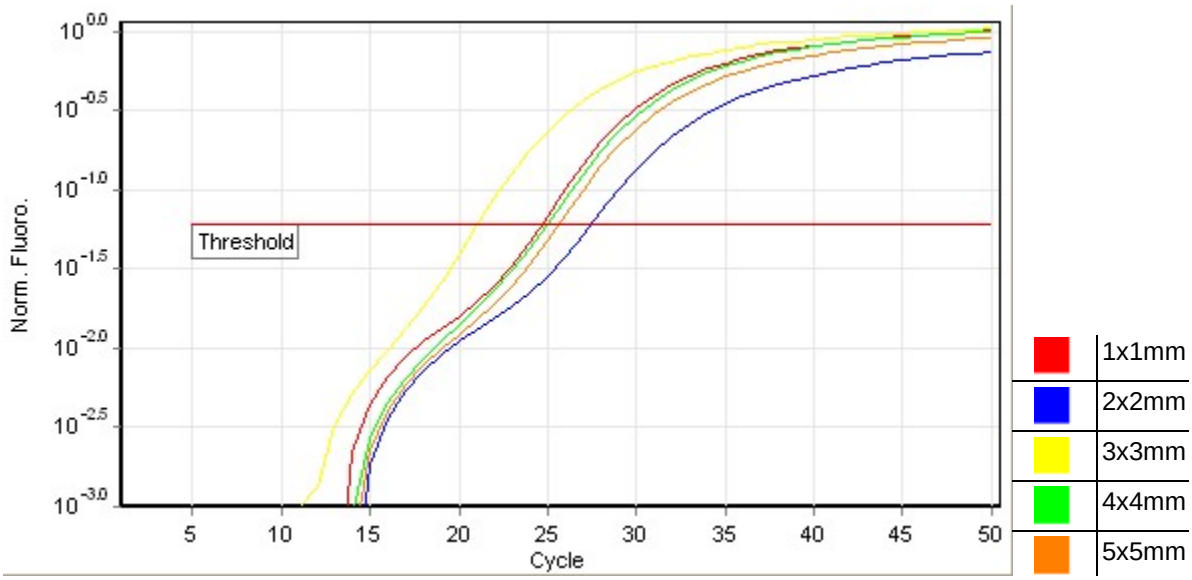

Figure 16. Expression of PRM2 in aged seminal fluid samples extracted by the AllPrep Kit. After room temperature storage for 1068 days, dried semen stains (1x1 – 5x5 mm) were extracted using the AllPrep DNA/RNA Mini Kit. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the PRM2 Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

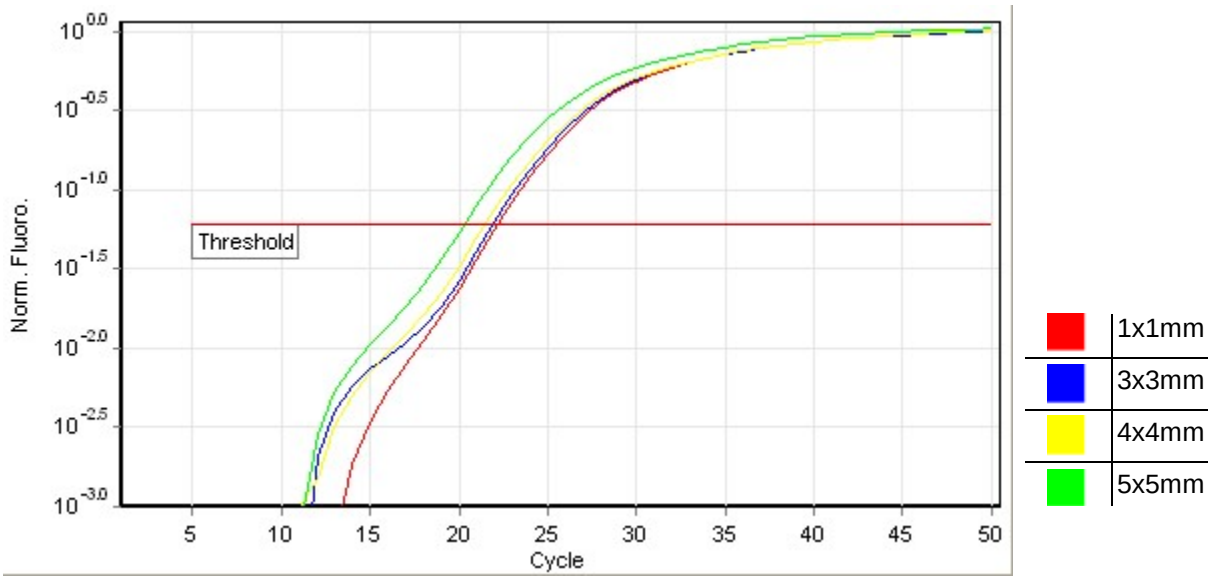

Figure 17. Expression of PRM2 in aged semen samples extracted by the Absolutely RNA® Kit. After room temperature storage for 1068 days, dried semen stains (1x1 – 5x5 mm) were extracted using the Absolutely RNA® Kit. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the PRM2 Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

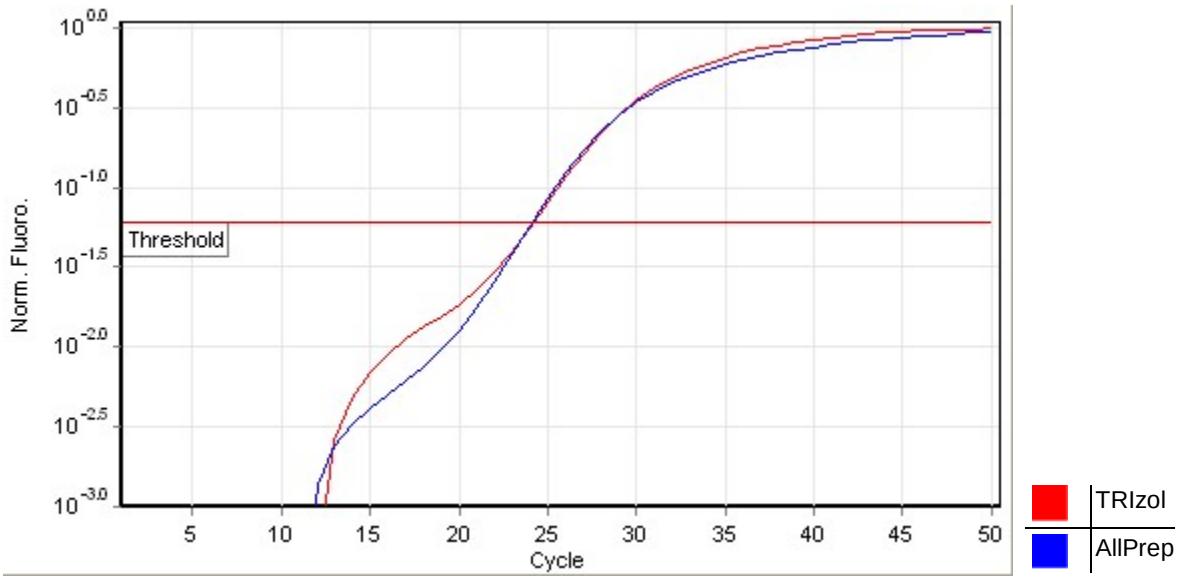

Figure 18. PRM2 expression in 4 year-old semen stains. After room temperature storage for 1667 days, dried semen stains (5x5 mm) were extracted using the TRIzol® reagent and AllPrep DNA/RNA Mini Kit. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the PRM2 Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

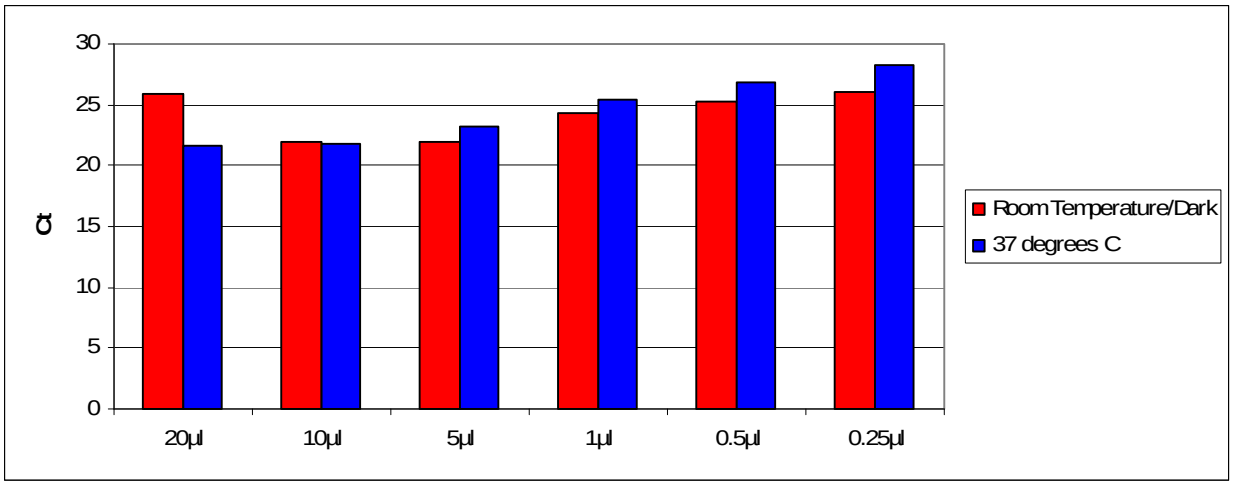

Figure 19. Effect of storage conditions on HBB amplification from blood stains. After room temperature or 37˚C storage for 503 days, dried blood stains (0.25 – 20 µl) were extracted using the TRIzol® reagent. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the HBB Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

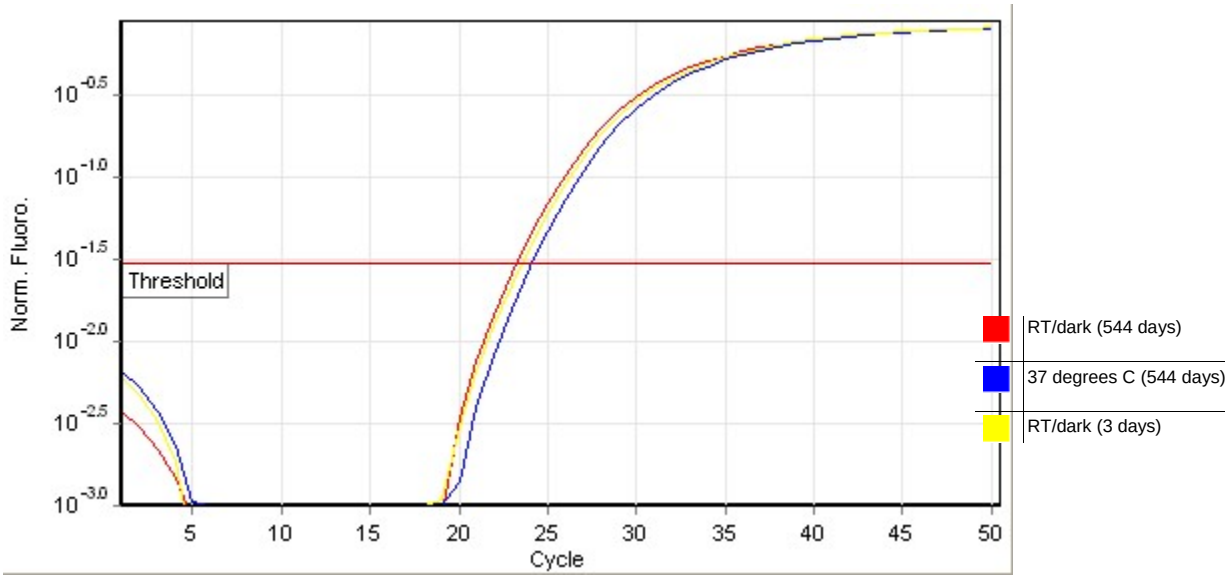

Figure 20. HBB expression in aged blood stains stored at various conditions. After room temperature or 37˚C storage for 3 or 544 days, dried blood stains (1 µl) were extracted using the TRIzol® reagent. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the HBB Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

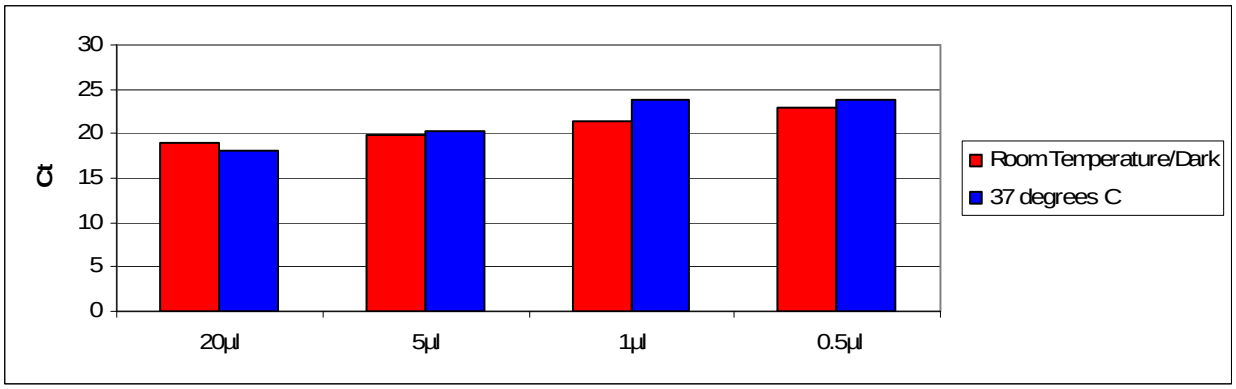

Figure 21. Effect of storage conditions on PRM2 amplification from seminal fluid stains. After room temperature or 37˚C storage for 518 days, dried semen stains (0.5 - 20 µl) were extracted using the TRIzol® reagent. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the PRM2 Assays-on-Demand™ Gene Expression Product. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

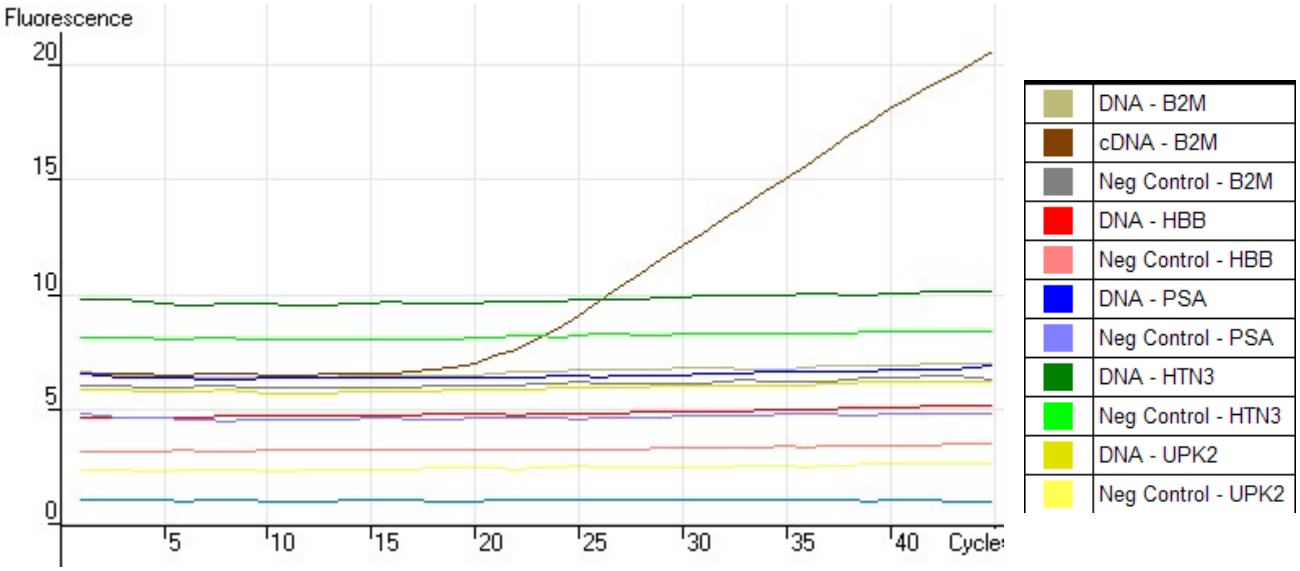

Figure 22. DNA and cDNA amplified with five cDNA-specific assays. After room temperature storage for 18 hours, a 20 µl blood stain was extracted using the TRIzol® reagent. Six µl of RNA was reverse transcribed using the MessageSensor™ RT Kit. Two µl of the resulting cDNA was used with the B2M Assays-on-Demand™ Gene Expression Product. Water as a negative control or control DNA (Promega; 256 ng in a 10 ul reaction) was used with the B2M, HBB, PSA, HTN3 and UPK2 Assays-on-Demand™ Gene Expression Products. Real-time PCR reactions were performed on a Corbett Rotor-Gene 6000 and data analyzed using the Rotor-Gene Software.

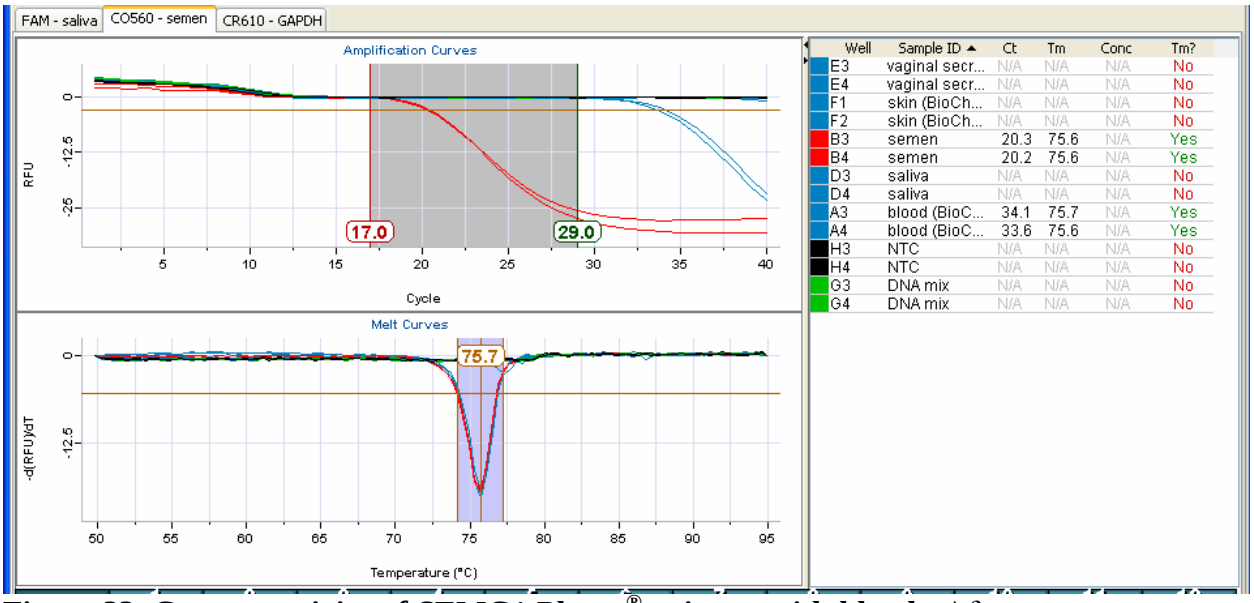

Figure 23. Cross-reactivity of SEMG1 Plexor® primers with blood. After room temperature storage for 18 hours, dried vaginal secretions, semen and saliva stains were extracted using phenol-chloroform or the Promega PureYield RNA midiprep kit (vaginal secretions). The RNA was quantitated and 100 ng of RNA was used in the Plexor® Stain ID assay. Control skin and blood RNA (BioChain) was also analyzed. Reactions were performed in duplicate on a Corbett Rotor-Gene 6000 and data analyzed using the Plexor® analysis software.

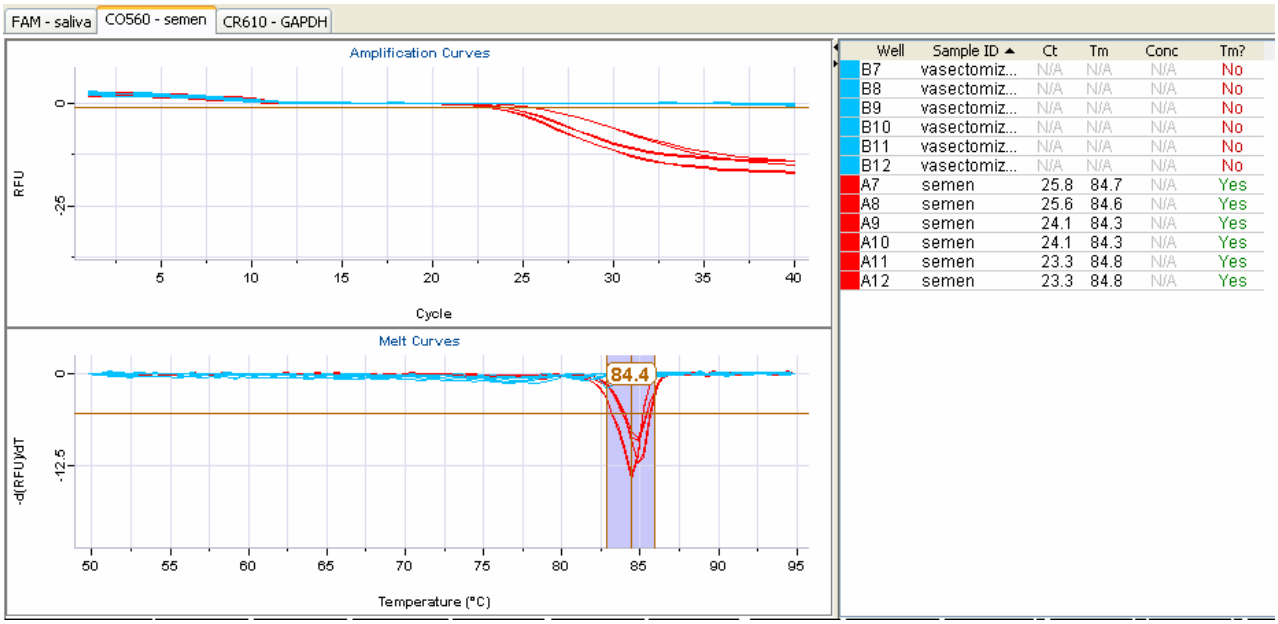

Figure 24. Absence of PRM2 expression in vasectomized males. After room temperature storage for 18 hours, dried semen stains from 6 individuals were extracted using phenol-chloroform. The RNA was quantitated and 100 ng of RNA was used in the Plexor® Stain ID assay. Reactions were performed in duplicate on a Corbett Rotor-Gene 6000 and data analyzed using the Plexor® analysis software.

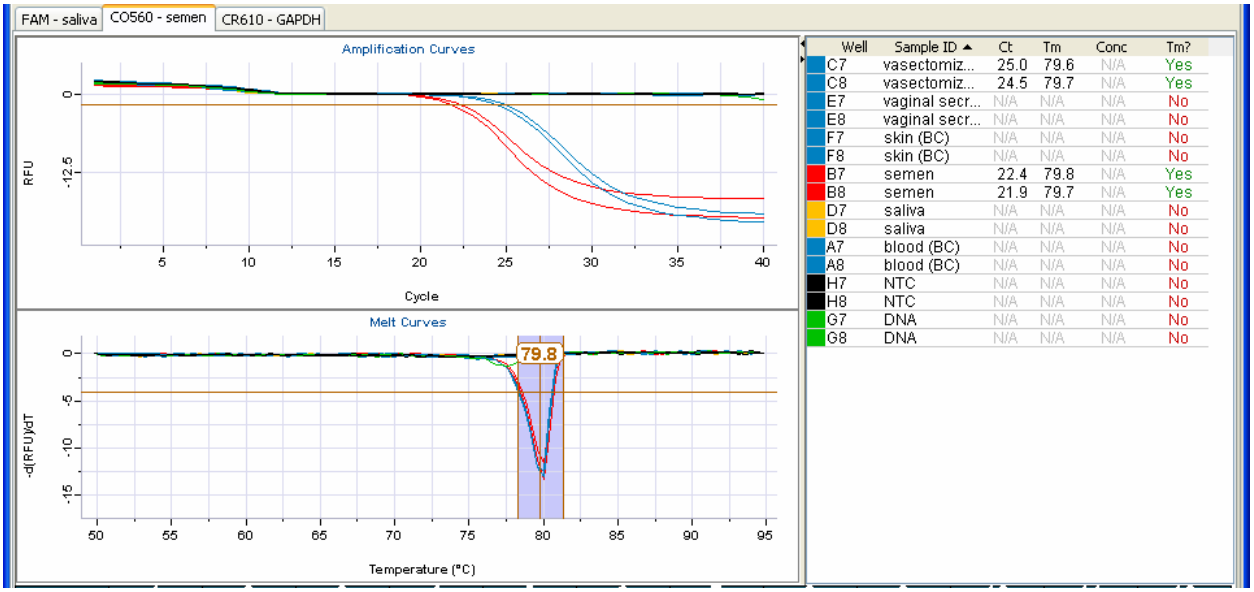

Figure 25. Specific expression of TGM4 in semen samples. After room temperature storage for 18 hours, dried vaginal secretions, semen and saliva stains were extracted using phenol-chloroform or the Promega PureYield RNA midiprep kit (vaginal secretions). The RNA was quantitated and 100 ng of RNA was used in the Plexor® Stain ID assay. Control skin and blood RNA (BioChain) was also analyzed. Reactions were performed in duplicate on a Corbett Rotor-Gene 6000 and data analyzed using the Plexor® analysis software.

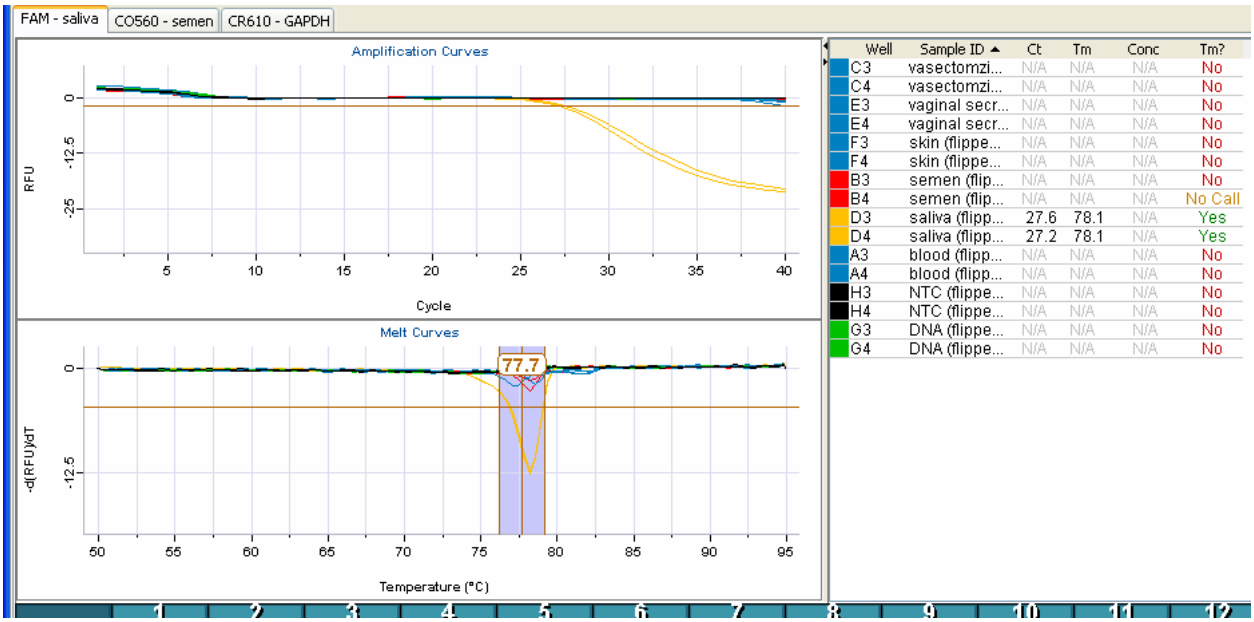

Figure 26. Modification of HTN3 primers increases specificity for saliva RNA. After room temperature storage for 18 hours, dried vaginal secretions, semen and saliva stains were extracted using phenol-chloroform or the Promega PureYield RNA midiprep kit (vaginal secretions). The RNA was quantitated and 100 ng of RNA was used in the Plexor® Stain ID assay. Control skin and blood RNA (BioChain) was also analyzed. Reactions were performed in duplicate on a Corbett Rotor-Gene 6000 and data analyzed using the Plexor® analysis software.

IV. Conclusions

1. Discussion of Findings

The first goal of this project was to identify the best method to extract mRNA from a wide variety of stain types. Additionally, we sought to identify an extraction technique that would simultaneously extract DNA in addition to RNA. This was a major objective since the purpose of the stain identification assays is to determine whether a sample is worthy of STR analysis. Therefore, it is critical that the same sample extract be profiled since nearby stains may be of different origins. Furthermore, for small, limited samples, there must be sufficient sample remaining to obtain a profile or the RNA analysis may prove to be a waste of time.

Throughout this grant, we assessed a number of commercial extraction kits for their ability to extract RNA (and DNA). The most reliable RNA extraction method for all stain types tested was the TRIzol® reagent. This straight-forward procedure is cost-effective and provides high yields of quality RNA. However, there are disadvantages to using this method; it is labor-intensive (involves separate RNA and DNA steps) and provides poor DNA yields. Therefore TRIzol® is not recommended for limited samples.

Several commercial kits were purchased in order to assess the capacity to isolate RNA (and DNA) in terms of yield and amplifiability. The RNA (and DNA) extracted by these kits was compared to parallel samples extracted via TRIzol®. The High Pure RNA Isolation Kit, AquaRNA Kit, AllPrep DNA/RNA Mini Kit and Absolutely RNA® Miniprep Kit were tested. Although TRIzol® remained the gold standard for RNA extraction from stains, both the AllPrep

and Absolutely RNA® Miniprep Kit provided comparable yields of RNA, whereas the AquaRNA and HighPure kits faired poorly. In addition, the Absolutely RNA® kit was also successful at extracting RNA from human tissues. However, a major disadvantage of the Absolutely RNA® kit is its inability to co-isolate DNA.

Neither of the co-extraction methods (TRIzol® or AllPrep kit) was capable of matching the DNA yields obtained with the gold standard Organic method, but in most instances, the TRIzol® reagent and AllPrep kit provided sufficient amounts of DNA to perform downstream STR analysis.

A significant amount of time was spent on optimization of the Sucrose method as a co-extraction technique. Unfortunately, after an exhaustive amount of work, we were unable to increase the quantity and/or quality of RNA extracted via this method. However, through these experiments we were able to develop, optimize and validate a new DNA extraction technique which has led to significant savings in terms of analyst time and reagent costs. Also, we were able to rule out a number of commercial kits that were recommended extraction methods. Based on this work, future stain identification experiments will utilize the TRIzol® method and Absolutely RNA® kit for RNA extraction, as well as the AllPrep Kit for dual extraction of RNA and DNA.

A preferable DNA extraction technique should be simple, efficient, provide DNA of sufficient quality and quantity to be utilized in subsequent procedures, and ideally, involve non-hazardous reagents. The findings in this grant demonstrate that the Sucrose Method meets each of these criteria. The method is essentially two steps, using a buffer composed of safe chemicals, and yields DNA sufficient for downstream applications. An important consideration for many labs is

the cost of the extraction method. Table 27 shows a time (hands-on plus incubations) and cost comparison for the three extraction methods, indicating the Sucrose Method costs 2- or 3-times less than the Organic or BioRobot® M48 Methods, respectively. The majority of the costs associated with the Sucrose extraction are tips required for multichannel pipettors, a sound investment considering the savings in analyst time. In addition to saving time and money, the Sucrose Method does not require special equipment (i.e. robot) or unique supplies. Therefore, any lab should be able to implement this procedure with materials that are already on-hand.

Presently, Vermont convicted offender samples processed for CODIS are extracted using the Sucrose Method. The two most recent batches of samples processed using this technique saw 93% and 94% success rates from extraction to upload into CODIS.

The second goal of this project was to evaluate the stability of RNA in different types of stains. For over 5 years we have been collecting samples for the sole purpose of determining the stability of candidate mRNAs for stain identification. In order for a candidate to be implemented into a stain identification assay, it needs to be detectable for years following deposition of the sample. It was long believed that RNA was very unstable and difficult to work with, requiring dedicated equipment and laboratory space. However, due to the development of new techniques and the recent increase in knowledge concerning RNA, it has been shown to be relatively stable. Various groups have demonstrated the stability of RNA in forensic stains by traditional RT-PCR and more recently, RT reactions coupled with real-time PCR (Juusola and Ballantyne, 2003; Alvarez et al., 2004; Bauer et al., 2003; Nussbaumer et al., 2006). Furthermore, Setzer et al. (2008) recently addressed concerns on the stability, and hence recoverability, of RNA in forensic

samples (blood, saliva, semen, and vaginal secretions) exposed to a range of environmental conditions from 1 to 547 days.

In support of these findings, we used amplification of HBB and PRM2 genes as an indicator of mRNA stability in blood and semen samples, respectively. Regardless of the sample size (i.e. down to 1 µl), we were able to detect these genes in samples aged well over 3 years. In fact, PRM2 was amplified from RNA extracted from the first sample collected for this project, a seminal fluid stain aged for 1666 days at room temperature in the dark. In fact, it appears as though PRM2 expression may even increase over time, perhaps due to the loss of an inhibitor.

Since forensic samples are often exposed to a variety of environmental insults, we stored parallel blood and semen samples under ideal conditions (room temperature, dark) as well as in a sub-optimal condition (37˚C). By comparing gene amplification between the samples, we hoped to determine whether RNA profiling would be feasible for weathered forensic stains. There was no considerable decrease in the degree of amplification after prolonged exposure (>500 days) to elevated temperatures. These results further demonstrate how stable HBB and PRM2 RNA are over time. In conclusion, our results together with work published by other groups (Juusola and Ballantyne, 2003; Alvarez et al., 2004; Bauer et al., 2003; Nussbaumer et al., 2006; Setzer et al., 2008) prove that RNA can be recovered from forensically relevant biological stains in sufficient quantity and quality for mRNA analysis.

A major goal of this grant was to identify 2-3 gene candidates which were specific for each tissue of interest. The gene candidates utilized through the course of this grant were identified through surveys of the literature including PubMed, Gene and other databases. Initially we

identified 2-3 genes that appeared to be specific for each tissue. These screening studies were performed using TaqMan® primer/probe sets from Applied Biosystems because they were pre-designed, inexpensive and already optimized for their intended target. For each target, the sensitivity and specificity for the body fluid of interest were assessed. Once the assay was shown to be robust, we tested it on mRNA isolated from other fluids and tissues to demonstrate that the assay is specific.

A sensitivity study of the three candidate blood assays (HBB, CD3G, SPTB) demonstrated how minute volumes of blood could be detected using mRNA profiling. Amplification with the HBB probe/primers occurred with as little as 0.0001 µl of blood. Although the seminal fluid genes were not as sensitive as the HBB assay, amplification using the PSA and PRM2 TaqMan® sets occurred with 0.01 µl of semen. The lower detection limit for TGM4 was not determined, but assumed to be lower than the 1 µl tested based on the Ct since it was far from nearing Cts typically observed in negative samples. In an attempt to identify the lower detection limits of our seminal fluid assays, known quantities of a control testes sample were used. Both PRM2 and TGM4 were detected in 1 ng of RNA, whereas MSMB and SEMG1 were detectable at 10 ng and 100 ng, respectively. Although our goal of developing screening assays does not involve quantitation of our samples, this experiment demonstrated that TGM4 and PRM2 are robust candidates for the detection of semen or sperm.

The sensitivities for saliva candidate markers (STAT, HTN3) were not as low as found for the blood and semen assays; 1 µl for STAT and 5 µl for HTN3. However, the result is significant because there are no Cts observed for the negative controls. The two urine TaqMan® assays

(REN and UPK2) were unsuccessful using 20 µl urine stains. Therefore, other potential candidates need to be identified for urine.

Our diverse sample bank was used to assess the specificity of the candidate tissue-specific genes. These samples included blood, semen, saliva, menstrual blood, vaginal secretions, kidney, colon, adipose, skin, and control RNAs (brain, heart, liver, kidney, intestine). The fluid results were fairly straight-forward. HBB, SPTB and CD3G (blood markers) only amplified in blood samples, whereas STAT (saliva marker) only amplified in saliva samples. There was some cross-reactivity with the semen candidates. Some minor amplification occurred with vaginal secretions (SEMG1 and ACPP) and blood (ACPP). But, for the most part, the assays only amplified from semen stains. Based on these studies PSA, TGM4 and PRM2 were the most specific.

On the other hand, the specificity studies using the tissue assays were much more complicated. For all but one of the tissues (heart), there was cross-reactivity with nearly all of the candidates tested. The most challenging tissues to identify specific markers for were kidney and liver. Furthermore, even when some candidates appeared to be specific based on the results using the control RNAs, differing results were seen with actual human tissue samples. Such was the case with intestine and kidney genes. All 3 intestinal markers amplified solely from the control intestine sample, yet amplification occurred with human colon and kidney tissues. Similarly, CLCNKA amplified with only the control kidney RNA, yet was picked up in each of the human tissues tested. Therefore, before any definitive conclusions can be drawn regarding the brain, heart and liver assays, analysis using actual human tissues must be performed.

A major aim of stain identification using mRNA expression profiling is working towards multiplexing the real-time PCR assays once mRNAs are identified that clearly define specific types of stains. Since these assays are designed to function more qualitatively than quantitatively, a test of a single stain should typically give amplification with only one candidate. It is possible that a mixture could give several amplifications. However, it is of greater concern to know that a mixture does exist, than to know the exact amount of each fluid present.

Work from Juusola and Ballantyne (2005) identified tissue-specific genes for the more common forensic casework samples: blood, saliva, semen and vaginal secretions. They developed a multiplex RT-PCR assay composed of eight genes (SPTB, prophobilinogen deaminase [PBGD], STAT, HTN3, PRM1, PRM2, human beta-defensin 1 [HBD1] and mucin 4 [MUC4]) which could be analyzed on a standard capillary electrophoresis platform. However, we sought to develop multiplex assays which could be performed more efficiently using real-time PCR; a technology currently used in most forensic laboratories.

The Plexor® system from Promega was one technology we employed to develop multiplex assays. Our initial focus was to design a blood-semen stand alone assay using two blood-specific (HBB, CD3G) and two semen-specific (PSA, PRM2) genes. While the HBB primer sets worked well to amplify from blood samples, there was no significant difference in the degree of amplification between semen samples and the no template control for the PSA primers. We redesigned the PSA primers, but had a problem with amplification of genomic DNA even though the primers were designed to span an intron. The PRM2 Plexor® primers worked extremely well to amplify from semen samples with virtually no amplification from the non-semen samples or negative controls. Unfortunately, we were unable to optimize conditions for the other blood

marker, CD3G, so we didn't pursue it as part of the blood-semen assay. As a 3-plex assay, we were able to show that these primer sets discriminate their target RNA in a heterogeneous sample of blood and semen RNA extracts. Although this assay has yet to be tested and optimized for mock casework samples, we feel that it is a promising screening tool for blood and semen identification.

A second multiplex assay which we designed based on the Plexor® technology was a quick, one-tube seminal fluid assay, since seminal fluid analysis is a major task for all forensic laboratories. In two published studies, Juusola and Ballantyne (2005; 2007) have developed multiplex assays which include two genes per tissue for the identification of four fluids including semen; the idea being that the use of two markers for each tissue would improve assay specificity and provide analytical redundancy by taking into account possible biological variation in gene expression levels between individuals. The two semen-specific genes reported in these studies are PRM1 and PRM2. However, as shown in Figure 24, neither of these genes will be detected in vasectomized males. Therefore, any stains produced by an azoospermic donor would not be detected by these assays. For semen, we were interested in genes specific for sperm and prostatic components. Often, it is important to determine if semen is present even if the male is sterile or has had a vasectomy (i.e. no sperm). In other cases, it is important to know if sperm are present. An assay that could determine whether the stain 1) is seminal fluid and 2) contains sperm could alleviate the need to perform extensive microscopy for the identification of sperm.

During the course of optimizing the seminal fluid assay, we designed primers for ACPP (semen), PSA (semen), SEMG1 (semen), PRM2 (sperm) and GAPDHS (sperm). Often, the initial monoplex results were promising, but in multiplex we would see non-specific amplification. We

tried various combinations of primer pairs to produce different multiplex sets by combining the three different dyes which had been successful: CAL Orange 560 (PSA, ACPP), CAL Red 610 (SEMG1, PRM2) and FAM (GAPDHS). The triplex which worked the best was ACPP, PRM2 and GAPDHS; one semen- and two sperm-specific markers. All three primer pairs amplified from semen and a 1:1 mixture of blood and semen to a greater extent than blood alone or the negative controls.

In order to generate a multiplex consisting of four targets, we decided to order the PSA primers labeled with Quasar 670 (CY5), as opposed to the original label of CAL Orange 560 since the ACPP primers are also labeled with CAL Orange 560. As mentioned previously, we saw amplification of genomic DNA with these primers, so we DNase treated our samples which worked to decrease the amplification in the samples where the RT enzyme was absent. However, the addition of a DNase treatment step is one to be avoided since it adds a 30 minute step, often results in a loss of RNA, and should not be used in close proximity to evidentiary DNA samples. The multiplex consisting of ACPP, PSA, GAPDHS and PRM2 primer sets is promising. Further optimization of the assay is needed, but all four primer sets appear to work in the presence of each other to discriminate between semen and non-semen samples.

A third Plexor®-based multiplex assay arose out of collaboration with Promega to develop stain identification assays. The long-term goal of the collaboration is to generate a panel of Plexor® Tissue Typing Systems; multiplexed qRT-PCR systems for determining the source and quantity of a variety of human stains and/or tissues. The systems would include Plexor® primers for the detection of tissue-specific mRNA transcripts associated with semen, sperm, blood, menstrual blood, saliva, etc. By limiting the system to two-color detection (i.e. FAM and HEX detection),

it is compatible with the majority of real-time thermal cyclers currently in place in forensic laboratories. We sought to include controls (e.g. a housekeeping gene) or multiple, tissue-specific targets using the ability of the Plexor® Data Analysis Software to distinguish two different amplicons in the same dye channel, based upon thermal melt properties.

The Stain ID assay which we have developed to date detects semen and saliva. Plexor® primers were designed to amplify the targets TGM4 (semen), HTN3 (saliva) and GAPDH (housekeeper). Through numerous optimization studies, we generated saliva primers that show specific reactivity with saliva RNA and no cross-reactivity with other RNA samples or with genomic DNA. The semen primers (TGM4) show specific reactivity with semen RNA and no cross-reactivity with other RNA samples or with genomic DNA. Although initial studies were performed using RNA extracted from large sample volumes, unlike what's encountered in routine forensic work, titration experiments were performed and indicated that semen had a detection threshold of approximately 10 pg RNA whereas the threshold for saliva was approximately 1 ng of RNA. Furthermore, we assessed how the Stain ID assay would perform with typical forensic samples (i.e. various volumes of unknown RNA yields). The only non-specific amplification that occurred was from the HTN3 primers and the vaginal secretions sample. Since this experiment was performed, the HTN3 primers have been modified and no longer detect vaginal secretions.

A major outcome of the Stain ID assay development was the generation of a co-isolation method for RNA and DNA extraction. A combination of the RNagents Total RNA Isolation System (Promega) with a Tris-buffered phenol protocol was optimized for simultaneous extraction of the nucleic acids. The final extraction method was shown to extract sufficient quantities of quality

RNA and DNA from 1 and 10 µl of semen and saliva demonstrating its utility as a dual extraction technique. Although this method involves numerous hands-on steps, it is faster than the TRIzol® method, and produces significantly better DNA yields. To date, it's the best co-isolation method we've tested in terms of yields and amplifiability.

Promega is in the final stages of generating a draft Technical Manual that will accompany alpha-testing of the Stain ID kit. We anticipate successful results from this round of testing which will lead into production of Stain ID materials that are made and QC'd by the manufacturing department at Promega. Once these are available, we will select and extract a large sampling group from our inventory for validation. This second round of testing with the manufactured materials will include testing samples that are of different ages, spotted on different materials (i.e. other than cotton cloth), and stored under various conditions. Furthermore, these samples will also be evaluated for DNA yields and generation of STR profiles. We hope that the successful completion of these validation studies will result in the development of the first commercial RNA-based Stain ID kit for the detection of semen and saliva in forensic samples.

A second strategy for multiplexing tissue-specific assays was with the TaqMan® technology. Since the Gene Expression TaqMan® assays from Applied Biosystems are incapable of multiplexing, as they are all labeled with the same dye, we set out to design our own TaqMan® probes and primers in hopes of developing several specific multiplex assays. The first multiplex we designed was a semen-sperm detection assay using the TGM4 (CAL Orange 560; HEX) and PRM2 (FAM) markers in addition to the housekeeper B2M (CAL Red 610; ROX). While each primer/probe set amplified from semen when alone in the reaction, the combination into a single multiplex reaction caused the dropout of TGM4 and B2M amplification. Future optimization of

this assay is required which will focus on changing the primer and probe conditions since PRM2 is expressed to a much greater extent than TGM4 or B2M. We believe that a simple real-time TaqMan®-based assay to screen for the presence of semen and sperm would be of great value to the forensic community.

The second TaqMan®-based multiplex assay we developed was for the identification of brain tissue using ADCY1 (FAM), GPM6A (CAL Orange 560; HEX) and the housekeeper B2M (CAL Red 610; ROX). Each primer/probe set amplified from brain both when alone in the reaction, and in combination with the other primer/probe sets with no significant decrease in the degree of amplification when the sets were alone or combined into the multiplex. Further optimization is required to eliminate some minor amplification in the no template controls. Similar to the semen-sperm assay, this multiplex looks very promising for a brain screening assay. Importantly, we hope to obtain human brain samples from collaborators which would assist in our evaluation of this multiplex as a viable screening assay.

A third technology that may offer great promise of multiplexing to the forensic community is the Bio-Plex™ system which uses the multiplexing technology of Luminex Corp. to enable the simultaneous quantitation of up to 100 analytes. The QuantiGene® Plex Reagent System is one platform that can be coupled with the Luminex instrument to enable the simultaneous detection of multiple RNA targets directly from purified RNA. As a proof of principle, we ordered a 3-plex assay to detect PRM2, SEMG1 and B2M. The most exciting result was that samples prepared using a quick one-step lysis method fared better than samples where purified RNA was extracted using the TRIzol® reagent. As this multiplex assay was intended as a tool with which to evaluate the feasibility of this technology as a routine screening assay, we plan to design

future QuantiGene® Plex assays based on the needs of the community. A disadvantage of this platform is the number of steps it requires; not a significant amount of hands-on time, but numerous incubations and wash steps performed using a vacuum manifold. However, since the capacity of this system is much more than other real-time RNA-based multiplex assays, there is a potential to generate large screening assays not possible with other techniques. Therefore, the QuantiGene® Plex assays should not be used for simple multiplex assays, but for large assays not compatible with conventional thermal cyclers, real-time instruments or capillary electrophoresis systems.

2. Implications for Policy and Practice

The biochemical approach currently used in tissue identification has undergone some changes in the past few years but essentially still relies upon the same technology where selective antibodies detect antigens to a particular source. These approaches have been simplified to save analyst time but are limited in scope; many laboratories limit tissue identifications to blood and seminal fluid only. Other tissue sources such as saliva or vaginal fluid are implied but not truly identified. We believe that the positive identification of these and other tissues can be performed in a quick and efficient manner which would allow analysts to provide a better service, more efficiently. Also with this technique, we believe tissues that are currently not routinely evaluated could be easily assessed by all laboratories so that an equality of testing could be realized across the country. Presently, some biochemical or "serological" tests are only performed in a select few laboratories. Through the development of a universal approach to tissue identification, one could imagine a multitude of tests that could be carried out by anyone qualified to do any one of the tests. As such, a wide variety of tissues could be assessed and evaluated. As time progresses, the

courts and the forensic community itself will demand tests that truly identify a tissue and allow for a better understanding of the material composing a STR pattern. We believe that the evaluation of mRNA through real-time PCR will be a technique that can offer that level of confidence and expand our knowledge of the materials we routinely examine. An example of this need was realized recently in our laboratory. A small stain was detected on the muzzle of a revolver used in an apparent suicide; the weapon, however, was found in a separate room from the victim. While the STR profile of the stain matched the victim, it would have been very valuable to know if the stain contained brain tissue or was simply the victim's blood perhaps from an older, previous, unrelated cut.

We believe that in the next few years a transition from a conventional biochemical approach to a molecular biological approach will be realized which will replace routine tissue identification. Tests that are tissue specific and designed to be multiplexed could yield rapid results on minimal sample. Such testing could employ mRNA as the tissue-specific determinant. Research that moves this line of testing forward will be important to the forensic community and also to the criminal justice community in general.

3. Implications for Further Research

To date, over the course of this grant, we performed experiments to assess the specificity, sensitivity and discriminatory limits of real-time assays, as well as the stability of mRNA over time using various fluid-specific genes for blood and semen. Experiments to assess the stability of mRNA over time showed that mRNA isolated from blood or semen spots stored at room temperature for various amounts of time (up to 4 years) was amplified using the blood or semen

assays, respectively. In order to multiplex (in groups of 3-4) the real-time PCR assays, we designed Plexor® primers, TaqMan® probes/primers and QuantiGene® Plex assays in order to identify whether various tissues are present is a stain. Lastly, in the search for a dual DNA/RNA extraction method we optimized and validated (for CODIS samples and standards) an efficient protocol that yields quantities of DNA comparable to the current extraction methods of our lab.

While we have made significant progress during the course of the original grant, more time and effort is required to develop and validate full multiplex assays using the aforementioned technologies. A shorter-term goal is to further our efforts to find the best DNA/RNA co-purification method that yields sufficient material for both mRNA analysis and DNA profiling. Great strides have been made to this end through the development of a tris-buffered phenol extraction procedure. However, further studies are needed to optimize this method for multiple fluid and tissue samples as well as for mock casework samples. The extraction methods will need to be evaluated for different analysis platforms to ensure that the nucleic acids produced can successfully be analyzed. Additionally, the continued evaluation of appropriate genes for other fluids/tissues (urine, vaginal secretions, menstrual blood, skin, adipose, muscle, etc.) is necessary, as well as studies to assess mRNA stability, selectivity and suitability for forensic stain identification. As the cycle continues, once genes are identified for further fluids/tissues, multiplex assays for those genes must be developed.

Importantly, the tissue-specific genes must be evaluated using human tissue samples. Most of the studies presented in this report, with the exception of the fluids, kidney and intestine were performed with control RNAs. Although this provides a strong background for preliminary determination of specificity, it is not an accurate representation of how actual heterogeneous

tissue samples will react with the assays. Through collaboration with FAHC, we hope to obtain more samples in the coming months which include, but is not limited to, heart, brain and liver. Specifically, the TaqMan® brain assay which we are in the process of developing would benefit greatly from the brain samples.

To date, almost all of the samples we have worked with were spotted onto clean, cotton cloth. Although samples have been collected on different substrates (cardboard, carpet, vinyl, metal, etc.), they have yet to be analyzed to determine the stability of the RNA on various substrates. Furthermore, the effect of storage conditions on RNA stability was only superficially addressed in this project. Studies were conducted to assess the differences between room temperature storage and storage at 37˚C, but other environments encountered by forensic stains (sunlight, rain, soil, etc.) are yet untested.

The Stain Identification assays proposed through collaboration with Promega may also be improved upon. Future kits may contain additional primer pairs, facilitating the detection and quantification of multiple sample types. For example, it may be possible to detect eight unique transcripts/targets using a real-time thermal cycler capable of four-color detection. This is possible by designing the assay to detect two different amplicons in each dye channel. Each amplicon could be discriminated based upon the thermal melt characteristics of the amplicon.

Other potential features include the inclusion of primers for genomic DNA targets and/or internal amplification controls. The included featured will be dictated by the degree of multiplexing supported by the real-time thermal cyclers. The inclusion of primers for genomic targets would allow the user to assess the probability of generating a useful genotype in subsequent STR typing

experiments (this may also be possible using the RNA targets). The inclusion of internal amplification controls would allow the end-user to assess the presence of amplification inhibitors. As previously noted, primers targeting ubiquitously-expressed transcripts could serve as controls for RNA integrity.

Altogether these studies indicate that DNA and RNA can be co-extracted and the RNA fraction used in multiplexed real-time PCR assays. The development of real-time PCR assays to detect tissue-specific transcripts for human fluids and tissues is the focus of many laboratories. These assays can ultimately be multiplexed for faster determination of tissue origin. A major advantage to these assays is that a single sample extract will be used to classify the sample as blood, semen, vaginal secretions, brain, heart, etc. which will drastically reduce the number of identification tests performed prior to DNA profiling (if required). The realization of RNA-based profiling is in the immediate future, yet a large body of work remains to optimize these techniques for the vast number of sample types and conditions which are routinely encountered in the forensic laboratory.

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VI. Dissemination of Research Findings

While the stain identification assays we sought to develop would be useful in our own laboratory, a major goal of this grant was to distribute the information to the forensic community at large to improve criminal justice in the United States. To this end, we took a multi-tiered approach at disseminating our work. The first step was talking about our progress at scientific meetings which included, but was not limited to, the NIJ Grantees meeting, NIJ Conferences, the New England SWGDAM meeting, and the Promega Meeting:

Presentations at Scientific Conferences:

National Institute of Justice Seventh Annual DNA Grantees Workshop, Arlington, VA, June 2006; "Forensic Stain Identification by RT-PCR Analysis and Consequent Development of a New DNA Extraction Method", Oral Presentation

17th International Symposium on Human Identification, Nashville, TN, October 2006; "Forensic Stain Identification by RT-PCR Analysis and Consequent Development of a New DNA Extraction Method", Poster Presentation

Sixth Annual Advanced DNA Technical Workshop, East Captiva Island, FL, May 2007; "What, Where and How Much – DNA and mRNA Research in Vermont", Oral Presentation

National Institute of Justice Conference, Arlington, VA, June 2007; "Forensic Stain Identification by RT-PCR Analysis and Consequent Development of a New DNA Extraction Method", Poster Presentation

Applied Technologies Conference, Point Clear, AL, March 2008; "Simple, Real-time PCR-based Methods for Triaging Biological Evidence", Oral Presentation

New England SWGDAM Meeting, Concord, NH, March 2008; "VFL Research Updates on RNA-based Multiplex Assays", Oral Presentation

National Institute of Justice Conference, Arlington, VA, July 2008; "Development of RNA-Based Screening Assays for Forensic Stain Identification", Poster Presentation

19th International Symposium on Human Identification, Hollywood, CA, October 2008; "Development of an RNA-Based Screening Assay for Forensic Stain Identification", Oral Presentation

One approach to disseminating our results was to work with a company to produce our assays as kit(s) for sale to the forensic community. We forged a relationship with Promega and they are extremely interested in making stain identification screening kits based on their proprietary Plexor® method. They have provided us with Plexor® One Step qRT-PCR kits, positive control

RNA samples, and materials for RNA/DNA extraction. The will also be providing us with Powerplex 16 STR kits in order to verify that the DNA extracted with their recommended method is suitable for STR analysis.

The last way to disseminate our work was to publish our results in forensic journals. To date, we have published one article and we anticipate submitting at least one or two other manuscripts based on the results we have compiled during the course of this project.

Citations:

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