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On-site confirmatory test for tissue type and specimen age

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Abstract

The purpose of the research described in this report was to develop a relatively inexpensive, fast means of identifying body fluid type and to determine if the sample is of human origin. The ultimate goal of this work is to provide crime scene investigators with an assay that can be performed at the crime scene itself, allowing them to identify which samples to collect for DNA analysis and which ones to discard. Even if crime scene investigators elect to perform confirmatory tests at the crime lab, our approach provides a rapid and inexpensive means of simultaneously confirming up to three different body fluids from a single assay and if the sample is of human origin. DNA analysis of samples collected at crime scenes has played a critical role in numerous investigations. The importance of such analysis to the successful resolution of investigations has prompted investigators to collect an increasing number of samples potentially capable of yielding useful DNA data. The increased collection of samples has resulted in a considerable backlog of evidence. Although crime labs have substantially increased their ability to process samples, the year-to-year backlog continues to increase. Currently, presumptive tests are performed at the crime scene to determine if spots/stains might be a particular body fluid type and therefore may be of probative value. Samples that provide a positive response are then collected, cataloged, and stored until a lab technician can perform a confirmatory test to positively identify the body fluid type. The confirmatory tests themselves can be expensive and time consuming. Once a positive result is obtained, DNA is then isolated from the samples and the quantity of human DNA determined. We have utilized nanotechnology to develop a faster, inexpensive means of body fluid identification. Body fluid specific mRNAs were identified through literature searches and subsequently confirmed to be body fluid specific using PCR amplification. Molecular Beacons (MBs) were then designed to detect such body fluid specific mRNAs. MBs are short, single stranded nucleic acids capable of forming a stem and loop structure. The loop part contains sequences complementary to the targeted RNA while the stem is comprised of short sequences where the 3' and 5' ends of the molecule are complementary to each other allowing for intra-strand base pairing. At one end of the MB is a fluorophore while the other end has a quencher attached. While the 3' and 5' ends of the MB are in close association, due to the stem structure, the fluorescent molecule emits little detectable signal. In the presence of the targeted RNA, however, the loop portion pairs with the RNA, separating the fluorescing and quenching portions of the MB and allowing for generation of a positive signal. We have developed MBs to detect mRNAs unique to blood, semen, saliva, male blood, as well as one that is human specific and an internal control. We have shown that the MBs only detect the body fluid that they were designed to detect. This technique requires no conversion of the RNA into cDNA and no amplification step. In order to maximize the signal from the MBs, we have chosen Quantum Dots (QDs) as our fluorescing

reporter for all but the semen specific MB. QDs provide a much stronger signal than most/all older fluorescing molecules. We are in the process of multiplexing the various MBs such that in a single assay, all three body fluid types can be positively identified and the sample determined to be of human origin or not. We estimate that up to sixteen samples could be processed in one hour or less. The identification of which samples to collect and process at the crime scene itself will result in much faster processing of samples, reduce the number of non-informative samples collected which will hopefully help reduce the backlog of biological evidence awaiting processing. The results reported here represent a proof of concept for the feasibility of our approach. More optimization and a better definition of the parameters under which the assays are reliable (for example impact of the age of samples) need to be completed before implementation in the field.

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Executive Summary

The Problem:

Crime labs in the US and elsewhere have experienced a substantial increase in the number of DNA-related evidentiary samples that must be collected, stored and processed resulting in a significant backlog of samples awaiting analysis. Although crime labs are processing more samples each year, the backlog continues to grow. Much of the increase in sample collection can be attributed to the probative value of biological samples in identifying who was present at a crime scene. Crime scene investigators are cognizant of the fact that jurors, as well as lawyers and judges, expect DNA data whenever possible. Thus more and more samples of potential probative value are being collected. Presumptive tests are performed at the crime scene on spots/stains that might yield useful DNA evidence; if a positive result is obtained, such samples are collected, processed, and stored for future analysis. Typically, each body fluid type must be tested for separately, often involving laborious, time-consuming techniques. A positive presumptive result, however, does not guarantee that the collected sample is of the body fluid type tested for or that the sample is human in origin. Samples of non-probative value are often collected, cataloged, stored, and tested via confirmatory tests only to be discarded when such tests are negative. The collection and processing of such non-informative samples contributes to the workload and backlog of crime labs. A portable, rapid, and inexpensive means of performing confirmatory tests at the crime scene could potentially significantly reduce the backlog of evidence waiting for processing. Even if crime labs elect to perform our techniques in the crime lab, they represent a significant savings in both time and resources over what is currently available.

The Purpose:

We are seeking a technique that will rapidly and inexpensively determine if samples found at a crime scene are of a particular body fluid type (blood, semen, or saliva) and if the sample is of human origin or not. Ideally, the technique could be used either at the crime scene or crime lab to rapidly and efficiently determine multiple body fluids simultaneously saving both time and money. The ability to perform such confirmatory tests at the time of collection will allow for more rapid processing of evidence and the elimination of spurious samples, saving resources, time, and should help to eliminate the DNA backlog. Even if samples are not processed until examined in a crime lab, our technique is a fast, inexpensive means of confirming body fluid type.

Research Design:

Tissue and body fluid specific mRNAs have been identified through numerous studies, both by biomedical and forensic scientists. Such body fluid specific mRNAs are targets for identifying the nature of the spot/stain, knowledge of which can be useful in reconstructing events associated with a crime. A search of the literature revealed several candidate mRNAs that were purported to be unique to blood, semen, or saliva and we verified their unique nature by PCR analysis. Previous work using RNA to identify body fluid type has largely focused on using some variant of PCR amplification (as we did in the verification portion of the work) to detect the presence of the target RNA. Such procedures necessitate the conversion of the single-stranded RNA into double-stranded copy DNA (cDNA) followed by PCR amplification. PCR-based analysis can be expensive and time consuming, taking a day or more to complete. We examined a version of nanotechnology known as Molecular Beacons (MBs) as a non-enzymatic means of identifying the presence of body fluid specific RNAs in samples. MBs are single-stranded nucleic acid molecules capable of forming a stem and loop structure. The loop portion is complementary to sequences in the targeted RNA while the stem is comprised of short sequences at the 3' and 5' end of the molecule that are complementary to each other and capable of base pairing. At one end of the MB is a fluorophore capable of emitting light when excited and at the other end is a quencher that will capture such emitted light if it is within close proximity to the fluorophore. In the absence of the targeted RNA, the stem portion of the MB keeps the fluorophore and quencher close to each other so little light signal is emitted. In the presence of the target RNA, the MB opens up, base pairs with the targeted RNA, separating the fluorophore and quencher such that a positive signal can be obtained. Because the loop portion is much longer than the stem portion, the MB-RNA hybridization is much more stable than the intra-molecular stem structure. The hybridization takes approximately ten minutes to complete.

Many fluorophores are available for constructing MBs. We selected Quantum Dots (QDs) for most of our MBs because they give some of the strongest signals obtainable and multiple QDs are available that fluoresce at different wavelengths, making multiplexing of MBs possible. Functional, conjugated Molecular Beacons will henceforth by referred to as MB. 2'-O-Methyl-RNA oligonucleotides with a 3' Iowa Black quencher and a 5' biotin linker were obtained from a commercial source. The QDs were attached to the oligonucleotide via streptavidin-biotin linkage. The exceptions to this approach were for the two semen-specific MBs where we had commercially (Integrated DNA Technologies) synthesized MBs with Cy5 as the fluorophore. Cy5 fluoresces at a wavelength that will make multiplexing with the QDs easier.

MBs were designed to detect mRNAs that we had confirmed were unique to blood, semen, or saliva as well as one that (based on database searches and sequence comparisons)

should only detect human RNA, and an 18S rRNA as an internal control. The efficiency of quenching was determined by comparing fluorescence of the unconjugated QD to the conjugated QD. The efficiency of the conjugation was determined by how much fluorescence is obtained in the flow through after conjugation (QD that is not linked to the MB will be present in the flow through).

Purified RNA from the different body fluid types has been tested with each of the MBs to determine if false-positive signals are obtained. A one-way ANOVA and subsequent Tukey-Kramer HSD multiple comparison procedure were performed on the QD-MB data to identify any statistical differences. A similar statistical analysis with the Cy5-MB will be conducted. Total RNA present in the samples was determined using a nanodrop spectrophotometer to obtain the 260/280 spectral ratio. Dilutions of the RNA samples were generated to determine the sensitivity of the MBs.

Multiplexing of the four (and possibly five) different MBs will be done by adjusting the relative concentrations of the different MBs such that unique, clearly discernible emission spectra can be obtained for all MBs. For the semen-specific MBs, we chose a more traditional fluorophore, Cy5, which fluoresces at a wavelength away from those for QDs making multiplexing easier to obtain. The quencher and fluorophore were attached to our MBs commercially (Integrated DNA Technologies) so the efficiency of conjugating and quenching could not be determined.

Findings and Conclusions:

MBs were successfully developed that recognize blood-specific, semen-specific, and saliva-specific mRNAs. No detectable cross-hybridization was obtained ($p < 0.001$) with the MBs conjugated with QDs nor was there detectable cross-hybridization with the semen-specific MBs (statistics not completed). The MB for blood only gave a positive signal in the presence of blood derived RNA. The same held true for both the saliva MB and our redesigned semen MBs. Our original “semen-specific” (for RNA hHk1) MB turned out to be male-specific for blood as well as semen. The literature reports that the hHK1 alternative splice junction used to generate the MB is found only in semen. Our PCR analysis as well as original MB studies used blood and saliva RNA obtained from females. When the hHK1 MB was tested on male derived blood RNA, a very robust signal was obtained. Blood RNA from three males and three females were examined and all of the male derived RNA produced a strong signal while none of the female derived RNA provided a signal. This discovery sent us back to the literature to identify true semen-specific candidates for MBs. Two were identified, for RNAs SEMG1 and TMG4. MBs were developed and have been shown to only produce a strong signal in semen and not blood or saliva derived from either males or females. Thus, splicing of the hHK1 RNA in blood is sexually dimorphic. Given the sexually dimorphic splicing of hHK1, a female-specific MB for blood should be obtainable.

We can envision situations where differentiating between male and female blood could be useful and expect to develop a female-specific blood MB.

MBs, therefore, could provide the crime scene investigator a means of identifying blood, semen, or saliva stains and determination of human origin at the time of sample collection (i.e., at the crime scene itself). The investigator would know which samples to collect for further processing and which ones to ignore. To perform the assay, an investigator would need a means of isolating the RNA, a portable spectrophotometer, a portable centrifuge, lap top computer, pipette, and minimal disposables such pipette tips and tubes. The easiest and most convenient way to isolate the RNA is with a commercial spin column kit. This would necessitate the use of a small centrifuge and a power source for a centrifuge such as an Uninterruptible Power Supply (UPS) for assays conducted at the scene of the crime. The items listed above are all found in any modestly equipped biology lab so body fluid determination could be easily and quickly performed with standard lab equipment. Once the MBs are multiplexed, in a single assay, investigators would know if a sample contains blood, saliva, or semen and if the sample were human in origin. We estimate the cost of having a portable lab equipped as described above to be between $10,000 and $16,000. The cost of the reagents for each assay we estimate to be $10 or less.

We estimate that 12-16 samples, and possibly more, could be processed in roughly one hour's time. The limitations on how many samples could be processed would be determined by the difficulty of recovering the samples and the number of centrifuge slots. The assay itself takes roughly ten minutes. At the end of the ten minutes, the investigator would know if the sample was of human origin, and if it contained blood, semen, or saliva or any combination of the three body fluids. We have shown that as little as 2 μl of either fresh semen or salvia and 4 μl of blood provide enough RNA for a robust signal with our MBs. We are confident that further optimization is possible to increase the sensitivity of the assay.

This work has confirmed the potential for MBs to be used for body fluid identification. More work, however, needs to be completed before MBs are ready for the forensic investigator. Additional optimization needs to be completed and they need to be multiplexed. Multiplexing four MBs has proven to be challenging, originally due to the very strong signal generated by the 18S rRNA MB. We have significantly diluted the 18S rRNA MB and we have switched the 18S QD for the saliva one. We are currently working on multiplexing the new MBs. The number of individuals examined with our MBs need to be expanded to demonstrate the universality of this technique.

We need to empirically demonstrate that our 'human specific' MB does indeed only detect RNA derived from a human source. DNA sequence data base searches of commonly encountered mammals as well as chickens and salmon, indicate the human-specific MB should

be valid. Confirmation of human specificity will need to be accomplished by testing commercially available total RNA (or RNA we isolate from fresh samples) from species most likely to be encountered at a crime scene (dog, cat, mouse, rat, cow, pig, horse, sheep, and chicken).

In summary, the potential for MBs to provide rapid and inexpensive confirmatory tests for blood, saliva, semen and human origin, both at the crime scene and in the crime lab, is significant. Other MBs could be developed to differentiate other body fluid types as well.

Molecular Beacons as a Means of Body Fluid Identification: Proof of Concept

Introduction

Crime laboratories within the United States are currently experiencing an increase in the number of DNA-related evidentiary samples presented to them for storage and processing. This increase may be due to multiple reasons, including the CSI effect among jurors, in which the general public expects DNA evidence to be presented at every trial regardless of the crime committed being violent in nature or not [Durnal, 2010]. This increase in sample load was documented by the National Institute of Justice in 2011 [Nelson, 2011]. The report discloses that although crime laboratories are processing a higher number of casework samples than ever before, there is still an increase in the number of cases that are backlogged every year.

One way to reduce the amount of annual backlog is to provide an improved and more rapid means of body fluid confirmation, eliminating extraneous samples from timely downstream STR analysis. At present, there is one presumptive test that can identify the possible presence of the three most common biological fluids found at the scene of a crime (blood, saliva, and semen): an alternative light source. While most presumptive tests can be performed while at the crime scene, confirmation that a piece of potential evidence is biological in nature, along with determination of tissue-type, is usually performed within a laboratory setting. Multiple confirmatory tests exist for both blood and semen. Confirmatory tests for blood include identification of blood cells under a microscope [Shaler, 2002], crystal tests such as the Teichman and Takayama tests [Shaler, 2002; Spalding, 2003], and ultraviolet absorption tests [Gaensslen, 1983]. Semen is usually confirmed by either the presence of Christmas Tree stained sperm cells in the sample [Watson, 2004] or by the presence of prostate-specific antigen (PSA), which will provide a positive result even with aspermatic males [Greenfield & Solan, 2003]. Saliva, at this time, does not have a reliable test for confirmation.

Although all of the aforementioned techniques are reliable, many are time consuming and/or specific to determination of one particular body fluid at a time. When these techniques are destructive, there is the potential for large amounts of precious sample to be consumed in preliminary testing alone, especially when the samples may be of mixed origin (multiple body fluids from one or more individuals). Whether samples are from a human source, as compared to the many instances where domestic animals have left behind biological fluids, is usually diagnosed in separate tests as well.

Here we present proof of concept for a new potential confirmatory test for the three most common and forensically significant types of biological fluids and determination of human versus non-human origin. The tests consist of ten minute assays that detect forensically novel

tissue-specific RNAs with the use of fluorescently labeled probes known as molecular beacons (MBs). With additional probes for both a positive control and a human-specific sequence of a housekeeping gene transcript, it can be known almost immediately which tissue type(s) a sample harbors, as well as if it is human in origin.

We are not the first to propose the use of tissue-specific RNAs in the identification of body fluid. Many have attempted to use mRNAs specific to blood [Juusola & Ballantyne, 2003; Nussbaumer *et al*. 2006, Zubakov *et al*. 2009, Hass *et al*. 2009], semen [Juusola and Ballantyne, 2003; Nussbaumer *et al*. 2006; Hass *et al*., 2009], saliva [Juusola and Ballantyne, 2003; Nussbaumer *et al*. 2006; Zubakov *et al*. 2009, Hass *et al*. 2009], menstrual blood [Bauer & Patzelt, 2002; Juusola and Ballantyne, 2003; Hass *et al*., 2009], and vaginal secretions [Nussbaumer *et al*., 2006; Hass *et al.*, 2009] in order to provide a means of confirmation of the presence of these bodily fluids. More recently, Hanson *et al.* has proposed the use of micro-RNAs for the identification of each of the aforementioned body fluids in forensic evidence [Hanson *et al*., 2009]. Although promising, the one common characteristic among these techniques is the use of Reverse Transcription Real-Time PCR (qRT-PCR). qRT-PCR is an extremely sensitive technique for RNA detection; however, it can be rather costly and time consuming. Our presented technique is distinctive in that it requires no conversion of RNA to cDNA, nor PCR of any kind. RNA extracted from a piece of potential evidence is directly combined with the MB probes, from which an increase in fluorescence should occur in the presence of one specific RNA only. The emitted fluorescence is detected by a portable fluorospectrometer no larger than a tissue box and powered by a USB connection to a laptop computer. Resulting fluorescent peaks indicate which fluid(s) is present in a sample.

The MBs used within our assay are single-stranded nucleic acid probes that possess a hairpin shape (**Figure 1**) [Tyagi & Kramer, 1996]. The probe portion of the MB is composed of bases that are complementary to a nucleic acid target, but that do not form a complex secondary structure of their own. The stem of the MB is made up of 5-7 nucleotides of complementary sequence designed to flank either end of the probe sequence. The 5’ end of the MB is linked to a fluorophore, while on the 3’ end, a quencher molecule is attached. As long as the MB remains in a closed conformation, emission from the fluorophore is quenched; therefore minimal fluorescence is detected. This “closed” conformation changes when the probe is in the presence of its complementary nucleic acid target, here our tissue-specific RNA. The probe will “open” to bind to its target, which provides adequate distance between the fluorophore and quencher for increased fluorescence to be detected.

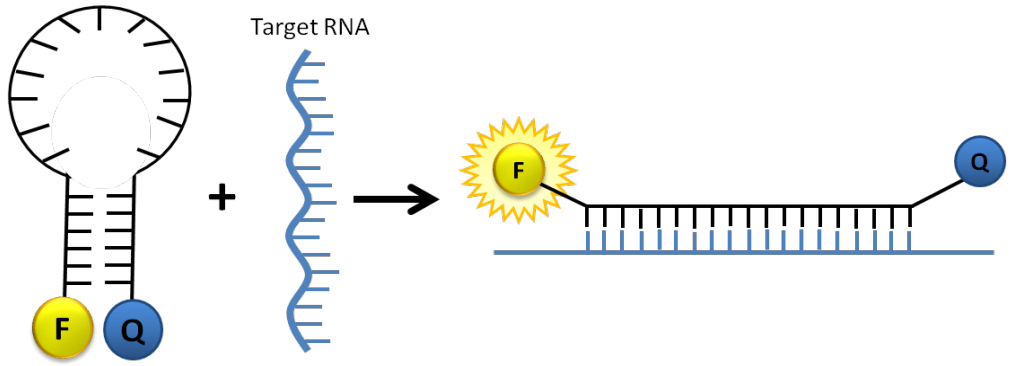

Figure 1: Structure and fluorescence of a MB in a "closed" and "open" conformation. When a MB is not in the presence of its target nucleic acid, it maintains a "closed" hairpin conformation in which the fluorophore (F) is being quenched by the close proximity of the quencher molecule (Q). Upon introduction of the target nucleic acid, here an RNA with a complementary nucleic acid sequence, the MB probe "opens" and binds to the target. Upon binding, the distance between the fluorophore and the quencher is great enough that increased fluorescence can be detected.

To avoid PCR, the fluorophore linked to MBs needs to be exceptionally intense. To fulfill this requirement, the fluorophore chosen was that of a Quantum Dot (QD) attached to the probe through biotin-streptavidin interactions. A QD is a commercially available nanocrystal composed of a core of CdSe molecules contained within a ZnS shell. These nanocrystals have a wide absorption wavelength, fluorescing under a wide range of light sources. The emission wavelengths are extremely narrow and are tunable based on the size of the CdSe core [Alivisatos, 1996]. Small QDs fluoresce at low wavelengths, while increasing the size of the QD increases the emission wavelength. This tunability allows for QDs of various diameters to emit different wavelengths, providing multiple fluorophores to later be used in multiplex reactions (one wavelength per blood MB, saliva MB, semen MB, and human MB). As previously stated, the intensity of QDs surpasses most other commonly used fluorophores. **Table 1** presents the extinction coefficients (used to determine intensity of a fluorophore) of two common fluorophores compared to two QDs with varying emission wavelengths. QDs are much brighter, but are still quenchable within in the MB form.

Table 1: Comparison of fluorophore intensity. Extinction Coefficients are listed for two common fluorescent molecules, FAM and Texas Red, as well as two QDs, one each from the upper and lower range of emission wavelengths. Commercially available QDs are anywhere from 5- to 168-fold more intense than many traditional fluorophores.

Fluorophore	Emission Wavelength (nm)	Extinction Coefficient ($M^{-1}cm^{-1}$)
FAM[Gene Link]	525	74,850
Texas Red[Gene Link]	583	136,000
QD525[Invitrogen]	525	710,000
QD800[Invitrogen]	800	12,600,000

The objective of this series of experiments is to produce a collection of MBs that detect tissue-specific RNA from blood, saliva, and semen, as well as a human-specific probe that can be used in an assay to confirm the identity of the type of body fluid found in evidentiary samples at crime scenes. For the purpose of this paper, the term “tissue-specific” will be used to describe any RNA found within, or probe that detects, only one of the three tissues tested. The authors recognize that the tissue-specificity of certain RNAs or probes could change upon further experimentation with additional tissue types. Candidate RNAs are either alternatively spliced or are tissue-specific due to epigenetic factors. Once RNA candidates are determined, the assay should have the potential to be used without PCR amplification of samples, to be multiplexed, and to be made portable to the scene of a crime.

It is believed that with further study, the assay will eventually be made to become portable to the crime scene itself, where it will allow crime scene technicians the ability to judge a particular piece of evidence as worthy of collection for further processing, or if it is not pertinent to the crime at hand. This, in turn, should help to reduce the number of samples collected at crime scenes, as well as reduce the sample load of backlogged crime laboratories.

Methods

Exploration and Rationale for Tissue-Specific RNA Candidates

To determine which RNAs should be examined for tissue-specificity, a literature search was performed. Multiple potential RNA candidates for blood, saliva, and semen were determined based on the criteria that each are: 1) highly expressed in a tissue of interest; 2) not expressed in any other tissue or tissues of forensic significance; 3) not a known marker for disease; 4) either alternatively spliced or harbor introns. Alternative splice sites provide a target area for the downstream creation of a MB probe that is tissue-specific based on splice pattern. If an alternatively spliced RNA could not be found, an RNA sequence that spans an exon-exon boundary can be used to make the MB RNA-specific. To determine if a suspected biological stain is human in origin, a control RNA that would be found in all three-tissue types, and in which a species-specific region could be targeted, was also sought. For this purpose, β-actin mRNA was candidate. Also, a control RNA was sought to act as a positive control in the determination that a piece of potential evidence was biological in nature. For this purpose, a highly conserved area of 18s ribosomal RNA (rRNA) was targeted.

The candidate tissue-specific RNAs featured in this manuscript were found in literature that utilized microarray or northern blot technology. The mRNA candidate thought to be specific to blood, erythrocyte membrane protein band 4.1 (4.1R), was chosen from the work of Yamamoto *et al*., in which microarray assays were used to illustrate the presence of certain alternatively spliced 4.1R mRNAs at various times during the lifespan of an erythrocyte [Yamamoto *et al*., 2009]. As for the saliva-specific mRNA candidate, epithelial membrane protein 1 (EMP1), Hu *et al*. employed mass spectroscopy and microarrays to try to characterize the diversity of RNA and proteins found in saliva [Hu *et al*., 2006]. From their supplementary data, multiple candidates, including EMP1, were examined for tissue-specificity. Finally, hexokinase 1 (hHK1) was selected as a semen-specific mRNA due to the work of Mori *et al.*, who used PCR-based screens and northern blots to investigate the sequence conservation across species of alternatively spliced type 1 hexokinase mRNAs thought to be testes-specific [Mori *et al*., 1996]. Serendipitously, we discovered that the semen-specific MB was in fact male-specific. Our early studies examined RNA from blood and saliva that were donated by a female. Upon examining blood derived RNA from a male, the 'semen-specific' MB gave a very robust signal. Examining blood derived RNA from three females failed to give a positive signal while blood derived RNA from three males all produced a robust signal. RNA processing in blood is therefore sexually dimorphic. No signal was detected in either sex's saliva. Using the same literature search and testing with PCR as before, we have identified two semen-specific RNAs, transglutaminase 4 (TGM4) (Lennard Richard *et al.*, 2012) and semenogelin (SEMG1) (Lilja *et al*.,

1989; Pang and Cheung ,2007; and Lindenbergh *et al.*, 2012), for semen MBs that do not produce a signal with blood or saliva RNA derived from either sex.

PCR-Based Screening of RNA Candidates for Tissue-Specificity

To investigate the tissue specificity of the selected target RNA molecules, a set of PCR primers was developed for each. The forward primer of each set was designed to bind to the RNA candidate at the site of the alternative splicing event that was thought to be the reason for the RNA's tissue-specificity. This is true for all targets except the 18s rRNA due to this RNA containing no introns.

If the RNA was not alternatively spliced, the forward primer would span the boundary of two exons, thus reducing the chance that any residual DNA in the RNA isolates would also be a target for amplification. The β-actin forward primer spans an area of the RNA in which the sequence is less conserved across species. All primer sequences used for PCR were designed using Applied Biosystems Primer Express Software version 3.0 (Foster City, CA). The sequences for these primers and the RNAs that they detect within each bodily fluid can be found in **Table 2**.

Total RNA was extracted from 15 μl blood, 30 μl saliva, and 30 μl semen sample aliquots using the SurePrep TrueTotal RNA Purification Kit (Fisher Scientific, Pittsburgh, PA) according to the manufacturer's protocol for each type of bodily fluid. All biological samples were obtained with a protocol approved by the West Virginia University Institutional Review Board for Protection of Human Research Subjects. The RNA isolates were reverse transcribed to cDNA for downstream PCR applications through the use of Applied Biosystems' Taqman Gold Reverse RT-PCR kit. A reverse transcription master mix was created with final concentrations: 1X Taqman buffer A, 2.5 μM random hexamers, 5.5 mM magnesium chloride, and 500 μM each dATP, dCTP, dGTP, and dTTP. 57 μl of the reverse transcription master mix was combined with 40 μl of the extracted RNA, 2 μl of RNase inhibitor (0.8 U), and 2.5 μl of Multiscribe reverse transcriptase (3.25 U). The samples were then pulse centrifuged, placed into a Techne Touchgene Gradient thermocycler (Burlington, NJ), and run under the following conditions: 25°C for 10 minutes, 48°C for 30 minutes, 95°C for 5 minutes.

cDNA from each of the tissues of interest was used as the template material for PCR reactions with each of the previously described primer pairs. Five microliters of cDNA was added to a PCR master mix with the final concentrations: 1 μM forward primer, 1 μM reverse primer, 12.5 μl of Applied Biosystems Universal PCR Master Mix, and nuclease-free water to bring the final volume of the mix to 20 μl. The samples were pulse centrifuged, placed into a Techne Touchgene Gradient thermocycler, and run under the following conditions: 1 cycle of

94°C for 5 minutes; 40 cycles of 95°C for 1 minute, 54°C for 30 seconds, and 72°C for 1 minute; 1 cycle of 72°C for 5 minutes. A negative control was also prepared for each PCR master mix that consisted of 20 μl master mix and 5 μl nuclease-free water.

PCR products and negative controls underwent agarose gel electrophoresis to observe the presence/absence of amplified bands for each tissue type. A 30 ml 1.5% agarose gel containing 1 μl 5mg/ml ethidium bromide was prepared for each primer set. Five microliters of PCR product was added to 5 μl water and 2 μl New England Biolabs Inc. Gel Loading Dye, Blue 6x (Ipswich, MA). Ten microliters of this mixture was added to each well. A 100 bp DNA ladder (New England Biolabs Inc.) was used as a reference. Gels were run in 0.5X TBE at 80V for 1.25 hours. Gels were visualized and photographed under a UV light source.

Table 2: PCR primer sequences used to detect presence/absence of RNAs from blood, saliva, and semen to evaluate tissue-specificity.

Bodily Fluid	RNA	RNA Alternatively Spliced?	Primer Sequence
Blood	4.1R	Yes	FP: 5'- AGCATGGAAGAAAAAGAGAGAAAGA -3' RP: 5'- TCTTCACCAGGGGAGGTCCT -3'
Saliva	EMP1	No	FP: 5'- ACCCTTCAGAACTCTCTTTGCTCA -3' RP: 5'- AACGCCGATGATGAAGCTG -3'
Semen	TMG4	No	FP: 5'- CATGGTGTAAAGAGGACATGGTTTT-3' RP: 5' GCACACATGGCCCTGCA-3'
Semen	SEMG1	No	FP: 5' ATGGGACAAAAAGGTGGATCA-3' RP: 5' GACTTTTTCGGGACTGGTCA-3'
Male-specific	hHK1	Yes	FP: 5'- GCAAGGACAGTGCGTTCAAGA -3' RP: 5'- CCGCATGGCATAGAGATACTTG -3'
Blood/Saliva/Semen (Human-Specific)	β-actin	No	FP: 5'- CCATCCATGTACGTTGCTATCCAGGCTGT -3' RP: 5'- GCAGCCGTGGCCATCTC -3'
Blood/Saliva/Semen (Positive Control)	18s	No	FP: 5'- CGGAGAGGGAGCCTGAGAA -3' RP: 5'- CTCCAATGGATCCTCGTTAAAGG -3'

Synthesis of Quantum Dot-Conjugated Molecular Beacons

Molecular beacons were synthesized according to established protocols for streptavidin-biotin-linked MBs [Cady *et al*., 2007] with two modifications: the use of 50kDal Amincon Ultra spin filtration system (Millipore Corporation, Billerica, MA) and the use of Incubation buffer (Invitrogen Corp., Carlsbad, CA) in place of PBS and BSA. 2'-O-Methyl-RNA oligonucleotides modified with an Iowa Black quencher on the 3' end and a 5' biotin linker were obtained from Integrated DNA Technologies (Coralville, IA). The sequence of each oligonucleotide was

designed to incorporate the complementary sequence of the forward primer used in the previous PCR-based screen. Six nucleotides of complementary sequence were designed to flank either end of the oligonucleotide to create the stem portion of the MB. Streptavidin-coated ITK QDs were obtained from Invitrogen Corp. The sequence for each MB oligo, the emission wavelengths of the QD label, and the corresponding quencher molecule used for each body fluid can be found in **Table 3**.

To link the ITK QDs to the MB oligos, different concentration combinations of QDs and MB oligos were tested. The concentration that provided the greatest quenching within the linked QD-modified MBs for each target RNA are seen in **Table 4**.

Table 3: 2'-O-Methyl-RNA oligonucleotide sequences including the fluorophore to which it was conjugated. Note that QDs are named after the wavelength where they fluoresce. Different quenchers (FQ and RQ) quench at different wavelengths. Underlined portions of MB sequences represent the complementary nucleotides that will form the stem of the MB in secondary structure.

RNA	Fluorophore	Specific Iowa Black Quencher	MB Oligonucleotide Sequence
4.1R	585 nm	FQ	5'- CCCGGAUCUUUCUCUCUUUUUCUUCCAUGCUUCCGGG -3'
EMP1	605 nm	RQ	5'- CCCGGAUGAGCAAAGAGAGUUCUGAAGGGUUCCGGG - 3'
TGM4	Cy5, 662 nm	RQ	5' CCCGGACATGGTGTAAAGAGGACATGGTTTTTCCGGG-3'
SEMG1	Cy5, 662 nm	RQ	5' CCCGGAATGGGACAAAAAGGTGGATCATCCGGG-3'
hHK1	655 nm	RQ	5'- CCCGGAUCUUGAACGCACUGUCCUUGCUCCGGG - 3'
β-actin	565 nm	FQ	5'-CCCGGAACAGCCUGGAUAGCAACGUACAUGGCUGGUCCGGG-3'
18s	525 nm	FQ	5'- CCCGGA TTCTCAGGCTCCCTCTCCG UCCGGG - 3'

Table 4: 2'-O-Methyl-RNA oligonucleotide and ITK QD concentrations to produce MBs. The following concentrations of each component of the MBs were combined to produce a probe with the lowest background fluorescence.

MB	QD (pmol)	Oligo (μl of 500 μM solution)
4.1R	35	2
EMP1	40	2
hHK1	40	3
β-actin	40	2
18s	30	2

Effective Coupling and Quenching of MBs

To determine the level of quenching upon linkage of the QD to the 2'-O-Methyl-RNA oligonucleotides (containing the quencher molecule), the fluorescence of the MBs were measured (in the absence of target RNA) and compared to the intensity of a blank created from an equal volume of incubation buffer and QDs alone. Fluorescence in the form of Relative Fluorescence Units (RFU) for each MB was quantified using a NanoDrop 3300 (ThermoFisher Scientific, Pittsburgh, PA) fluorospectrometer.

MB Specificity Assay – RNA Extracts from Biological Fluids

Proper and specific binding of MBs to target RNAs was determined by combining the probes with RNA extracted from fresh blood, saliva, and semen samples. RNA was isolated from biological fluids using TRI-Reagent BD (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. Each resulting RNA pellet was rehydrated with the addition of 40 μl nuclease-free water (Fisher Scientific, Pittsburgh, PA).

Again, different concentration combinations of MBs and RNA were tested to see which would produce the highest RFU values when combined. The reaction concentrations for each MB can be found in **Table 5**. These volumes were added to 0.5 ml tubes and the final reaction concentrations were brought to 25 μl with incubation buffer.

Table 5: Reaction conditions for each MB and RNA.

MB	MB (pmol)	RNA (μl)
4.1R	2	20
EMP1	4	5
TGM4	2	20
SEMG1	2	20
hHK1	4	5
β-actin	2.5	10
18s	2.25	10

After a 10 minute incubation period at room temperature, 2 μl of each RNA-MB mixture was read to determine fluorescence in RFU on the NanoDrop 3300. The blank for each measurement was composed of the same concentration of MB with incubation buffer to make the final volume 25 μl. A negative control measured RFU of 2 μl incubation buffer.

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Statistical Analysis

A one-way ANOVA and subsequent Tukey-Kramer HSD multiple comparison procedure were performed on RFU values from MBs alone, as well as MBs in the presence of complementary and non-complementary RNAs to determine if there was a difference in the amount of fluorescence recorded for all but the new semen-specific MB (work in progress). The level of significance was determined using $\alpha = 0.05$.

MB Sensitivity Assay

To gain insight on the potential sensitivity of the MBs to RNA that is potentially damaged or degrading, as one may see in forensic evidentiary samples, a series of serial dilutions was created from the RNA of each biological fluid. Again, these samples were tested in 25 μl reactions as described above and RFUs determined with the NanoDrop 3300.

Human-Specificity of β-actin MB

To determine the human-specificity of the β-actin MB, first an NCBI Blast search [Chenna *et al*., 2003] was performed on the RNA sequence complementary to the probe portion of the MB oligonucleotide. Search parameters were set to megablast highly similar reference RNA sequences from all species, except *Homo sapiens*. The resulting Taxonomy Report was examined to find taxa that may provide a source of contaminating RNAs at a crime scene: dog, *Canis lupus familiaris* (NM_001195845.1); mouse, *Mus musculus* (NM_007393.3); rat, *Rattus norvegicus* (NM_031144.2); and pig, *Sus scrofa* (AY550069.1). The RNAs detected were of the β-actin gene for each respective taxa. Other taxa were chosen beyond the Blast search as possible sources of RNA from either pets or food items: cat, *Felis catus* (GQ848333.1); chicken, *Gallus gallus* (NM_205518.1); cow, *Bos Taurus* (NM_173979.3); lamb, *Ovis aries* (NM_001009784.1); and salmon, *Salmo salar* (NM_001123525.1). Human β-actin mRNA sequence data was also obtained from NCBI: *Homo sapiens* (NM_001101.3).

RNA sequence data was aligned using the online multiple sequence alignment software ClustalW2 (European Molecular Biology Laboratory – European Bioinformatics Institute, Hinxton, England). Species-specific nucleotide polymorphisms were detected between the β-actin RNA sequence from humans and other taxa of interest.

Results

PCR Screen of Tissue-Specific RNA Candidates

Candidate RNAs were preliminarily screened for specificity using PCR. The forward primer was designed to span the nucleotide sequence that was believed to make each RNA tissue-specific. Total RNA extracted from blood, saliva, and semen, after reverse transcription, was PCR amplified using each primer set. Results of the PCR products run on agarose gels for three-candidate tissue-specific RNAs, along with a candidate human-specific RNA, are found in **Figure 2**. Primers for 4.1R mRNA were designed to amplify a 266 bp region thought to be specific to blood. Results of gel electrophoresis display a single band when these primers were used in a reaction containing cDNA from blood. Amplification did not occur when 4.1R primers were used in PCR with cDNA from saliva or semen. Similar results were seen with primers designed to amplify EMP1 and hHK1 mRNAs. EMP1 produced the expected 498 bp band only in saliva, while hHK1 shows a 186 bp product only in semen, and subsequently male but not female blood (data not shown). 18s, the positive control for biological fluid produced a 171 bp product in all tissues examined. β-actin (ActB), the speculated human-specific primer pair, produced a 500 bp band of amplified product from cDNAs from all three tissue types as expected. Negative controls, in which nuclease-free water was substituted for cDNA, did not produce a band for any of the primers tested.

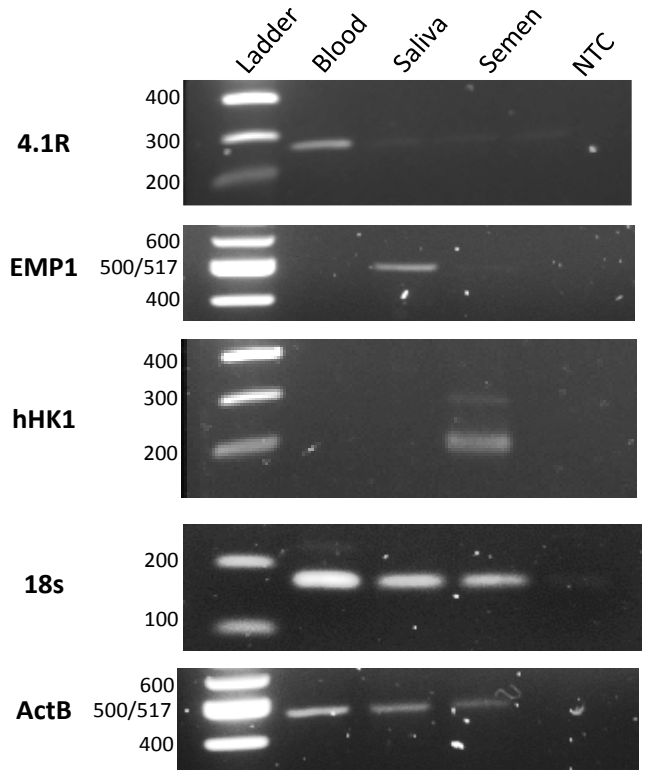

Figure 2: Tissue-specific RNAs as determined through a PCR screen. Total RNA extracted from blood, saliva (both from females), and semen was reverse transcribed. Resulting cDNAs were used as the template material for PCR amplification with primers designed to amplify possible tissue-specific regions of candidate RNAs. For the tissue-specific RNA candidates shown to the left, each band is of the expected length and amplifies RNAs found in only one of the three biological fluids tested. The β-actin primers were designed to be human-specific and were found in all three tested fluids. A negative control (NC) for each PCR reaction yielded no amplified product. Similar results were obtained for TGM4 and SEMG1 (data not shown).

Effective Coupling of QD and MB

After linking the streptavidin-coated QD to the biotinylated oligonucleotide sequence containing an Iowa Black quencher molecule, the amount of fluorescence the MB exhibits should decrease. Effective coupling of QDs and MB oligonucleotides is demonstrated in **Figure 3**. Levels of QD quenching vary, with the greatest decrease in fluorescence, 79.4%, displayed by the QD-MB for EMP1 RNA for detection in saliva (**Figure 3B**). The MB designed to detect hHK1 in semen (**Figure 3C**) is the least quenched with a 29.5% reduction in fluorescent signal. Subsequent analysis indicated that hHK1 not only detected semen but male derived but not female derived RNA from blood. The remaining MBs for human-specificity (**Figure 3D**) targeting β-actin, for 4.1R RNA in blood (**Figure 3A**), and for the positive control (18s) (**Figure 3E**) show a reduction in signal of 66.3%, 65.7%, and 55.1% respectively.

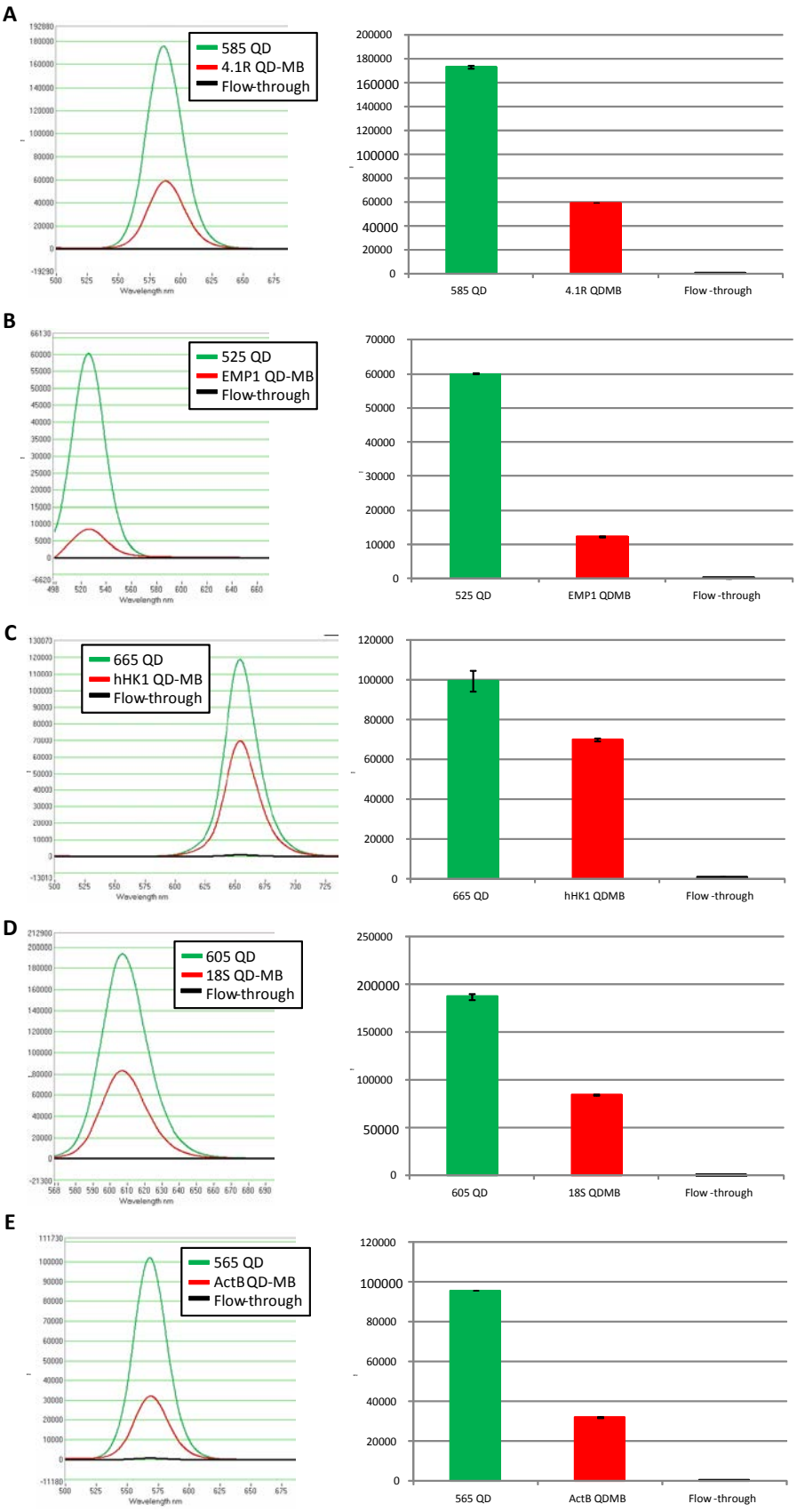

Figure 3: Quantified RFU of each QD versus QD-MB. The mean RFU values of each QD (shown in green) are compared to the mean RFU of the same QD once linked to form a MB (shown in red, QD-MB). The flow-through collected during synthesis is also shown (in black). MBs were designed to detect the presence of the candidate tissue- or human-specific RNAs (A) 4.1R, (B) EMP1, (C) hHK1, (D) ActB, and (E)18S. Error bars are standard error where n=9.

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Specificity of Tissue-Specific MBs

MBs were tested for specificity by combining each with RNA extracted from blood, saliva (both derived from females), and semen samples (**Figure 4**). An increase in fluorescence was seen when each tissue-specific MB was in the presence of RNA from only one biological material [blood (n= 7 people, 3 males and 4 females), saliva (n = 5 people) and semen-specific MBs (n = 2 people]. β-actin and 18s MBs, as expected, showed an increase in fluorescence when combined with RNA from all three tissue types. This illustrates proper function of the probes in which a conformational change is possible when the target RNA is present. When in the presence of RNAs from other sources, an increase in fluorescence was not detected. These results demonstrate that the MBs will not open in the presence of merely any RNA, but only when the target RNA is present.

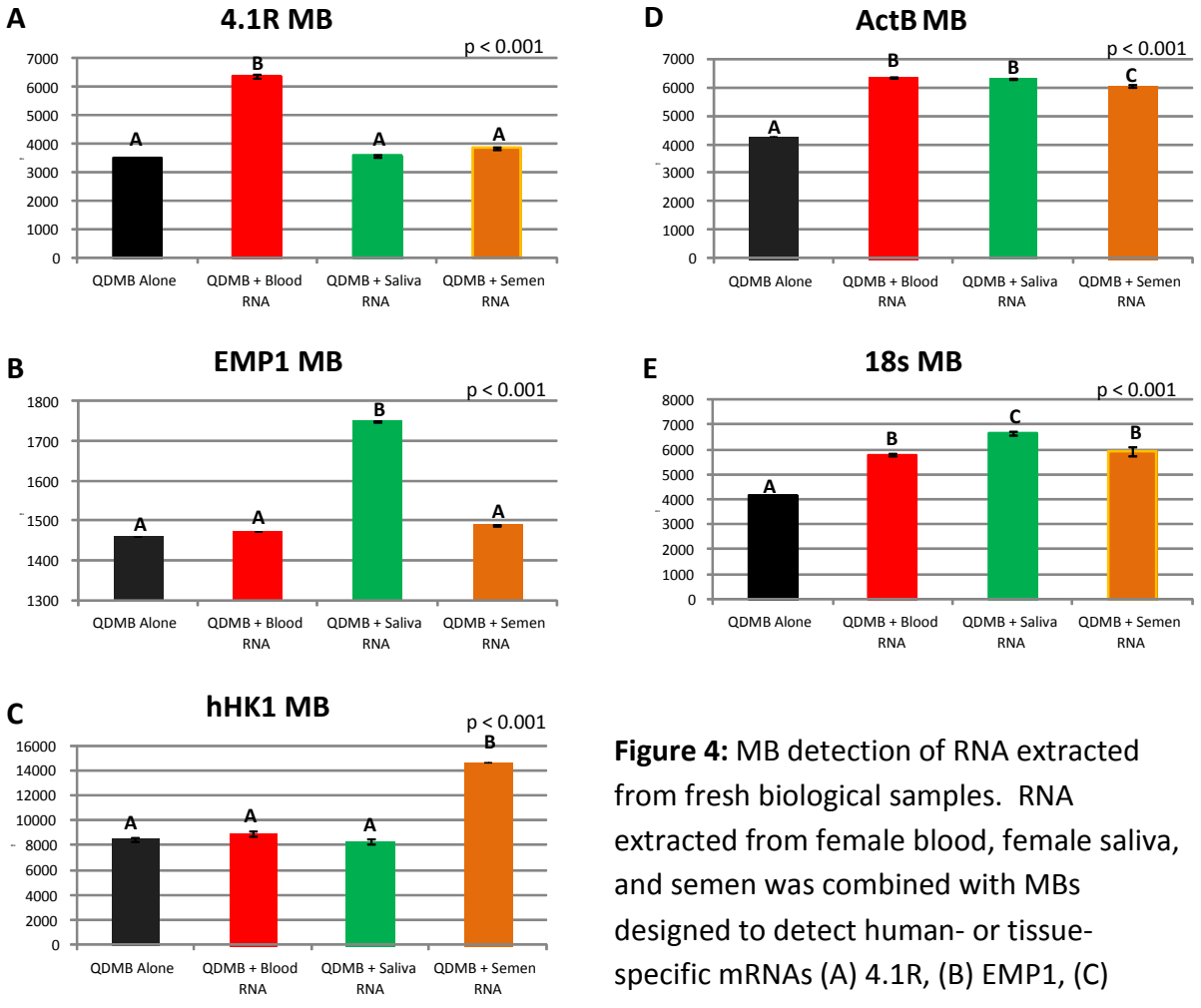

Figure 4: MB detection of RNA extracted from fresh biological samples. RNA extracted from female blood, female saliva, and semen was combined with MBs designed to detect human- or tissue-specific mRNAs (A) 4.1R, (B) EMP1, (C) hHK1, and (D) ActB, and (E) 18S. Error bars are standard error where n=12. P-values for each one-way ANOVA are present. Means associated with the same letter are not significantly different (Tukey-Kramer

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MB Sensitivity

In a series of 50% dilution tests using RNA from fresh samples, ranging from undiluted down to 0.0625 of starting material, we have demonstrated in singleplex reactions that MBs for both saliva and semen gave robust signals. The lowest amount of saliva in the reaction was 408 ng of total RNA (based upon 260/280 nanodrop spectral data). The original isolate came from 30 μl of saliva thus a 0.0625 dilution represents less than 2 μl of saliva. For the semen, as little as 245 ng of total RNA could be detected. As with saliva, the original sample from which RNA was extracted was 30 μl so we can identify as little as 2 μl of fresh semen. We did not do any dilutions below 0.0625 so our sensitivity may be even greater. We will determine the absolute minimum sample size needed for a positive signal. In the case of blood, we only obtained a robust signal with samples diluted down to 0.125 of original volume. This represents 940 ng of total RNA or the amount that would be obtained in 4 μl of fresh blood. We are working on making the blood test even more sensitive.

Specificity of β-actin MB

To better estimate the specificity of the β-actin MB, the human RNA complement sequence of the probe was compared to similar sequences of species possibly present at a crime scene. These similar sequences were determined by the results of an NCBI Blast search. Among the most highly similar sequences was the RNA of several taxa's β-actin gene. The alignment of all sequences, including the messenger RNA (mRNA) sequence of human β-actin, is shown in **Figure 5**. It was observed that the sequence of human β-actin mRNA differs from all other taxa at nucleotide positions 15 and 18. The salmon species β-actin RNA sequence differs most from human with a total of 4 mismatches, while the taxa of dog, mouse, and cat differ the least with 2 mismatched bases. The remaining taxa differ in sequence at 3 nucleotide positions, although the position of each base discrepancy is not the same in each taxa.

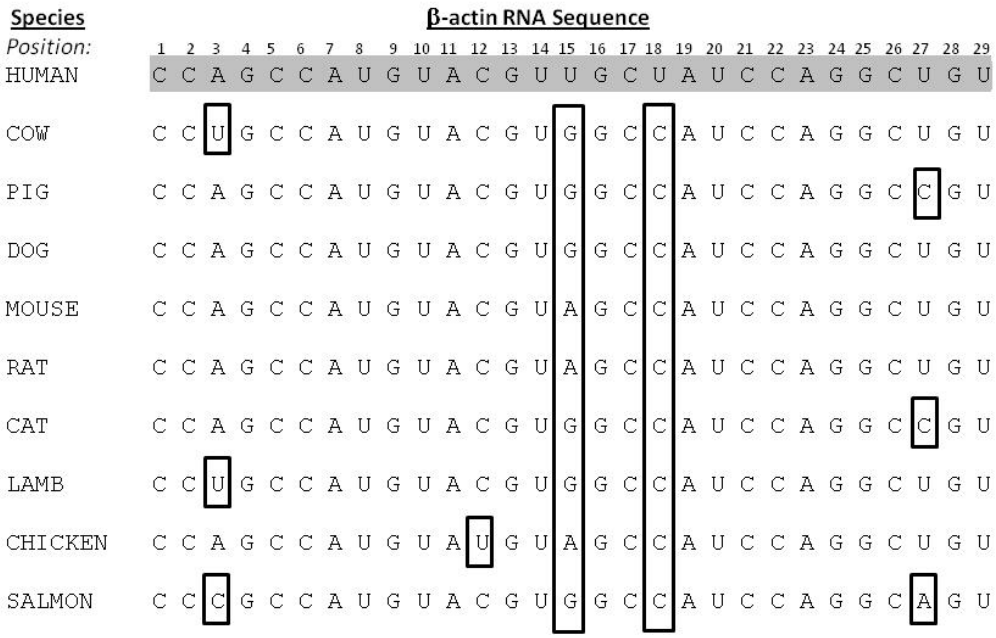

Figure 5: Sequence alignment of β-actin mRNA from multiple taxa. To determine species-specific sequence polymorphisms, the β-actin mRNA sequences of 9 taxa that may provide a source of contaminating RNA at crime scenes were aligned. All non-human taxa sequences were compared to human (highlighted in grey). Sequence aberrations from human are held within boxes. Each base is given a numerical nucleotide position to better reference its location.

Conclusions

The purpose of this study was to provide the forensic community with novel probes for later use in the confirmation of the presence of biological fluids in potential evidence found at crime scenes. Knowing if a stain is biological in nature, as well as determining exact tissue-type, while still at the scene can help to reduce the number of items collected by crime scene technicians. The results of this study confirm that tissue-specific RNAs can be targeted to determine the biological make-up of a stain without the use of PCR. The presented method, once optimized in multiplex, may help to reduce the amount of backlog that is currently plaguing crime laboratories nation-wide.

Multiple tissue-specific RNA candidates where sought in the literature. After testing 36 candidates, three RNAs were preliminarily determined to be specific to one of three fluids, blood, saliva, or semen, through the use of a PCR-based screen. A potential human-specific region of β-actin mRNA was present in all three tissue types. Although all candidate RNAs selected using this method did provide MBs that were tissue-specific, this assay was merely for preliminary purposes to screen possible MB sequences for further analysis. Unexpectedly, our original semen-specific MB gave a robust signal with RNA derived from male but not female blood. A reexamination of the literature revealed two additional candidates that are semen-specific.

The forward primer of the PCR-based screen was used as a template for the design of MB probes. The MB oligonucleotides were linked to their fluorophores, QDs, using biotin-streptavidin interactions. The MBs were tested for proper conjugation using an assay in which quenching of the fluorophore was measured. It was determined that all MB oligonucleotides containing a quencher molecule had been correctly coupled to the corresponding QD. The fluorescence of certain MBs was more highly quenched than others, exhibiting a range of 79.4% to 29.5% reduction in fluorescence. Although MBs for hHK1 and 18S showed the least reduction in quenching, further optimization can be managed. Using a lower concentration of QD and/or higher concentration of MB oligonucleotide with attached quencher in initial linkage reactions may help to further reduce the RFU of the "closed" MB. Both of these actions would provide QDs conjoined to multiple oligonucleotides, and therefore multiple quencher molecules. Although we have simplified the appearance of a MB in **Figure 1**, depending on the concentrations of the components used for coupling, the MB produced most likely takes the form of the probe seen in **Figure 6**. Streptavidin proteins coat the entire outer surface of the QD. Depending on the ratio of concentration of QDs to MB oligonucleotides in the initial reaction, several oligonucleotides could potentially bind to the QD, quenching the fluorophore further. This enhanced quenching will lead to an increase in the fluorescence detected when the MB is in the presence of its target RNA.

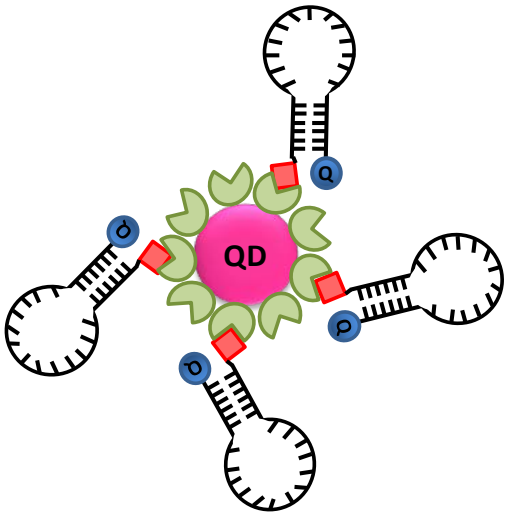

Figure 6: A more realistic View of QD-MBs produced through biotin-streptavidin coupling. Streptavidin proteins, shown in green, coat the surface of the QD. One biotin protein, shown in red, is attached to the 5' end of the MB oligonucleotide. The concentration of QD and MB oligonucleotide can be modified to maximize the number of oligonucleotides coupled to each QD. The more oligonucleotides attached, the further bright QDs will be quenched.

Although the proposed tissue-specific MBs were found to be specific to the RNAs isolated from only one type of bodily fluid (with the exception of the original "semen-specific MB) and the MBs targeting β-actin and 18s recognized all three human tissues, the amount of fluorescence detected in RFU differ between each MB (**Figures 4 and 5**). This is most likely due to the concentration of each RNA in the body fluid tested. Increased signals can be obtained by optimizing the amount of MB and isolated RNA used in each reaction. Further reduction of the fluorescence of the "closed" MB, by increasing the number of quencher molecules attached to each QD as previously described, may also increase the signal produced by the MB when in the "open" conformation. Furthermore, this difference in fluorescent signal strength may indicate that increased biological sample sizes of more than 30 μl of semen and saliva should be used for analysis. These variations of the original protocol should be the focus of future experimentation.

Although how a probe works in singleplex is important, the final goal of experimentation is to provide one, multiplex assay to detect the presence of all three of these fluids in one sample, as well as to determine if that sample is human in origin. Singleplex reactions provide some information on how these probes will work in multiplex, but much optimization will need to be performed in order for all of the probes to function at an optimal level once combined in multiplex.

The human-specificity of the MB for β-actin was examined by means of computer software analysis. A Blast search revealed several similar sequences from animals that could potentially provide a source of contamination at a crime scene. All of these sequences were of β-actin RNA. After sequence alignment, it was discovered that many of the animal β-actin RNA

sequences differed from human by 2-4 nucleotide bases. It was determined that the β-actin MB should have little difficulty selecting its target complement over the other sequences. This is due to the increased ΔT_m of MBs compared to linear probes, which is attributed to the thermodynamic properties of stem portion of the MB [Farjami *et al*., 1995]. It is also believed that 2'-O-methyl RNA MBs possess a superior affinity for RNA targets [Cummins *et al*., 1995; Tsourkas *et al*., 2002]. 2'-O-methyl RNA MBs will bind more quickly to an RNA target than to a DNA target. Those nucleic acid probes that have even less binding affinity than any 2'-O-methyl version are simple RNA:RNA or DNA:DNA hybrids. To be absolutely certain that the MB for β-actin will only target human RNA, the only reliable test is to use the probe in reactions with RNA isolated from each of the animals examined, or at least those most similar to the human sequence. If RNA from dog, mouse, or rat does not elicit a fluorescent response, it is not likely that RNA from any of the other animals will as well.

Potential Portability of Assay

Our focus has been on developing the techniques that could be used in either a crime lab or potentially a portable system that could be used at the crime scene itself. The techniques are the same. To make this approach portable, several needs must be met: Fluorospectrometer, small centrifuge, a stable surface upon which to place the centrifuge and fluorospectrometer, laptop, power source for the centrifuge, a means to isolate the samples, pipette, and reagents (maintained at room temperature).

Figure 7. A) fluorospectrometer B) small centrifuge

The fluorospectrometer shown in **Figure 7A** weighs 3.3 pounds, is approximately the height of a small Kimwipe box and shorter than a standard tissue box. It is powered by the laptop. This

fluorospectrometer is the NanoDrop 3300 and was used in all of our studies. It is available from Fisher Scientific at a list price of $12,320. Several small, relatively portable centrifuges exist. The one shown in **Figure 7 B** is from Neutec. It weighs between 10-15 pounds depending upon rotor, measures 8.2 x 9.6 x 5.7 inches (208 x 245 x 145 mm), can handle 12 2 ml tubes in a single spin and generates 12,300 g force. It is listed for ~$800. A relatively small platform/table to hold the centrifuge and fluorospectrometer could be obtained for less than $100 from numerous sources or the floorboard of a van should be sufficient. An adequate Dell laptop can be obtained for less than $500. Power for the centrifuge could be supplied by either local sources or a Universal Power Supply coupled with a power amplifier for $2,000. Various companies (for example, Fisher's SurePrep TrueTotal RNA Pur kit) sell simple, phenol-free spin columns for safe and quick purification of RNA at room temperature. Cost of reagents and disposables is estimated to be no more than $10-12 per sample.

Implications for Policy and Practice

The use of MBs at the scene of a crime to identify samples of likely probative value will result in substantial savings in time and logistics. Spurious samples will not be collected, cataloged, stored, and subjected to confirmatory testing. The elimination of uninformative samples should significantly reduce the backlog of biological samples waiting to be processed.

We believe that this technique will have multiple advantages over currently available presumptive and confirmatory tests for biological evidence. First, our technique has a short assay time, in which multiple samples can be processed in less than an hour. Without the requirement of PCR, this method features a reduced assay time, as well as reduced cost. Current technology utilizing qRT-PCR can cost $25 per sample. Our assay, at this time, with small-scale production of MBs, would cost approximately $10-12 per sample to complete. This cost is for a multiplex reaction, where any of the three biological fluids tested can be determined and, it is anticipated, determination of human origin, as well as the possible determination of the components of mixtures. Currently, at least two different tests performed in the laboratory would be needed to acquire both pieces of information. Although more tissues of forensic importance need to be tested for certainty, this technique has the potential to be the first reliable confirmatory test for saliva. Finally, the possibility for portability to the scene of a crime is high. Only a limited number of items are needed to run the assay and each is of small size and weight. A relatively small one-time investment of $10,000-16,000 will equip crime scene investigators with the necessary tools for identification of three different body fluids (and others could be developed) and confirmation of the sample being of human origin. The isolation of RNA and the MB assay are straightforward techniques that can easily be taught in an hour or two.

Implications for Further Research

Once optimized in a multiplex reaction, further experimentation will be needed to determine limitations of the MB technique due to the age or degraded state of samples. It is also necessary to determine the smallest volume of biological fluid required for proper confirmation of presence in a multiplexed assay. The number of individuals tested with our MBs need to be expanded. Identification of a female blood-specific MB is also desirable. Furthermore, it may be possible to remove the use of an RNA isolation technique that requires a centrifuge. The probes were composed of 2'-O-Methyl-RNA oligonucleotides. These bases are highly resistant to enzymatic degradation [Cummins *et al*., 1995] and may allow for an assay somewhat similar to that used for *in situ* hybridization studies. A biological sample would be collected and then combined with a lysis reagent to break open cells and release the RNA held within. The lysed cellular components would then be added to the MBs, where RNA is targeted and increases in fluorescence read directly from the tissue lysates. This type of RNA "isolation" procedure would significantly reduce total assay time, as well as eliminate the need of a portable centrifuge. Removing the need of a centrifuge improves the ease of portability.

Additional MBs could be developed to other body fluids that might be of interest such as menstrual blood and vaginal mucosa. Such MBs could also be used at the crime scene or used in a crime lab to determine body fluid type.

We are unaware of a company that routinely attaches QD to MB but companies will attach more traditional fluorophores to MB. Changing to commercially available conjugated MBs would be a significant savings of time and may not significantly reduce sensitivity of the assays. Future work could determine the sensitivity of such MBs as well as multiplex them into a single assay.

This preliminary study provides proof of concept for the use of MBs for tissue-specific RNAs in the confirmation of body fluids from potential evidence. Although further experimentation will be required before use on forensic samples, the presented method holds multiple advantages over current techniques. The advantages of time and cost alone would allow for our assay to replace current techniques in the laboratory if portability were to be deemed too cumbersome.

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Dissemination of Research Findings

Presentations of the research:

2011 On-site Body Fluid Identification. Young, S.T. and Bishop, C.P. American Academy of Forensic Sciences, Chicago, IL (poster)

2011 Portable Assay for Body Fluid Confirmation: Proof of Concept. Young, S.T. and Bishop, C.P. 19th International Association of Forensic Sciences, Funchal, Madeira, Portugal (talk)

2012 On-site confirmatory tissue type and specimen age. Young, S.T. and Bishop, C.P. National Institute of Justice Conference, Alexandria, VA (invited talk)

2013 Body Fluid Identification via Quantum Dot Molecular Beacons. Moore, J.R., Young, S.T. and Bishop, C.P. International Association of Bloodstain Pattern Analysis, San Diego, CA (invited talk).

A manuscript is in preparation for submission to a peer-reviewed journal.

The above document is a draft manuscript that will be submitted to a prominent forensic science journal such the Journal of Forensic Science. An additional manuscript is anticipated.