2015

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Expanding the Use of RNA in Forensic Science: A Quantum Dot Molecular Beacon Approach

Joshua R. Moore

Thesis submitted
to the Eberly College of Arts and Sciences
at West Virginia University

in partial fulfillment of the requirements for the degree of

Master of Science in
Forensic Biology

Committee Members:

Clifton P. Bishop, Ph.D., Chair
Stephanie T. Young, Ph.D.
Daniel G. Panaccione, Ph.D.

Department of Biology

Morgantown, West Virginia
2015

Keywords: Quantum dot molecular beacons, body fluid identification, forensics, RNA
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Abstract
Expanding the Use of RNA in Forensic Science:
A Quantum Dot Molecular Beacon Approach

Joshua R. Moore

As the popularity of shows such as *CSI* increases, so does the demand for DNA evidence in court cases. This has led to the overwhelming of capacity for analysis of biological samples at crime labs across the country, and to the development of a massive backlog of DNA evidence waiting to be processed. At the end of 2011, labs had a processing capacity of 250,000 cases per year, but an annual total of nearly 350,000 that needed to be processed. Such delays have effects throughout the US justice system. One way to reduce the backlog would be to increase the value of the evidence retrieved from the scene, reduce the collection of unnecessary evidence, and retrieve as much data from the evidence as possible in the fewest steps. We investigated a novel, confirmatory method of body fluid identification using quantum dot molecular beacons (QDMBs). The QDMBs target RNA species that are tissue-specific or sex-specific, allowing for the identification of body fluid stains and identifying the sex of the depositor of a bloodstain. Our results show that we were successful in developing QDMBs that identify blood, semen, saliva, male blood, and female blood, along with a human-specific beacon. We were also able to detect aged stains, although the age detectable is dependent upon the particular fluid tested. This technique has a low cost, confirmatory testing, and a high potential for portability, meaning it could be performed at the scene to increase the value of evidence retrieved. In addition to being a confirmatory test, it does not lead to the degradation of DNA, as can some presumptive tests. We believe this technique holds great potential for increasing the value of RNA in forensic evidence and helping to reduce the biological evidence backlog.

Acknowledgements

I would like to thank first Dr. Clifton Bishop, my advisor. You have provided much guidance and support personally and professionally, and I feel I have grown considerably as a scientist and a person during my time in your lab. While the research has had its ups and downs, I knew I always had your support. Special thanks go to Dr. Stephanie Young for starting the research on the molecular beacons, and giving me a solid foundation upon which to perform my research. I also wish to thank Dr. Daniel Panaccione as my third committee member, and a professor whose class I greatly enjoyed. Your guidance has been helpful, particularly as a voice from outside of the field of forensic science.

I would like to thank my lab partner Amanda Balasko, for her assistance on this project and her help as one of many donors that made the research possible. You have been a great support, and in training you I learned even more about the research.

In addition, I would like to thank the faculty, staff, and graduate students of the Department of Biology. None of this would be possible without you.

Finally, I want to thank my friends and family for all their support and love over the years. This has not always been an easy road, but I have always known I had your support.

Table of Contents

Title Page	i
Abstract	ii
Acknowledgements	iii
Table of Contents	iv
List of Tables	v
List of Figures	vi
Introduction	1
Methods	6
Analysis of Tissue-Specific RNA	6
Development of Molecular Beacons	6
Conjugation of Molecular Beacons to Quantum Dots	7
Analysis of Sample Using QDMBs	8
Specificity of QDMBs	8
Aging of Samples	8
Statistical Analysis	9
Results	10
PCR Verification of Body Fluid Specificity	Error! Bookmark not defined.
Effective Conjugation of MB to QD	10
Tissue Specificity of Conjugated QDMBs	10
Effectiveness Using Aged Samples	11
Discussion	16
Bibliography	19
Appendix 1: JMP Output	21

List of Tables

Table 1: Fluorescence values for organic flurophores versus quantum dots.	5
Table 2: RNA identities and primer sequences for body fluid identification.	9
Table 3: Body fluid specific RNA identities with QD wavelength, quencher, and beacon sequence.	9

List of Figures

Figure 1: A traditional molecular beacon and its functioning. GeneLink, 2015.....	5
Figure 2: Theoretical structure of QDMB.....	5
Figure 3: Relative fluorescence of QD versus QDMB.	12
Figure 4: Tissue specificity testing of QDMBs.....	13
Figure 5: A comparison of target and non-target QDMB fluorescence expression.....	14
Figure 6: Results of aging of samples, tested with tissue-specific beacon.	14
Figure 7: The JMP output for ANOVA analysis of the female-specific beacon testing data.	15

Introduction

The field of forensic science, and DNA analysis in particular, has grown substantially in recent years due to advancements in the technologies used for forensic analyses. Likewise, there has been an increase in the amount of evidence collected by crime scene investigators and thus an increase in the amount of evidence in need of processing by forensic laboratories. The amount of evidence collected and the amount of evidence that can be processed in a given period of time have both grown, but at unequal rates, leading to a substantial backlog of evidence waiting for processing (1). As this backlog grows, cases take longer to be investigated, trials are delayed, and the criminal justice system slows. As of 2011, US labs had the capacity to process almost 250,000 cases, but had a total DNA case load of almost 350,000 cases. Although the labs are increasing their capacity they are still being outpaced by the growth in demand for casework services (1).

There are multiple ways to reduce this backlog and increase the rate at which evidence is processed. Some methods include: increasing the employment numbers at crime laboratories to increase the volume that can be processed at a given lab; improving the techniques used to analyze the evidence such that results can be obtained in a more efficient manner; or, developing a method of ensuring only the evidence of highest value is retrieved from the scene and processed at the crime laboratory (1, 2).

This research explores a possible answer using that third method, of increasing the value of evidence retrieved and analyzed. If confirmatory testing is performed in the field, less analysis time in the lab will be wasted on preliminary steps, and processing time and effort will be saved. A portable technology that gives a confirmatory, rather than presumptive, answer to the identity

of a body fluid stain and confirms the presence of nucleic acids useful for further analysis would be of great value. This would increase the value of evidence retrieved and allow field investigators to reject samples that add little or no value to the investigation.

The focus of this research is on body fluid stains, as they are likely to contain DNA evidence that can be used to identify suspects and connect a suspect to the scene or to the victim. It is true that many tests for certain body fluids already exist, but there are not confirmatory tests for each body fluid (3, 4). Also, many of these confirmatory tests can only be performed in an equipped forensic lab and not in the field (4-7). Portable presumptive tests may give false positives, or worse, false negatives for body fluids. Additionally, some presumptive tests have negative effects on the stains, affecting the DNA results later in the process (8). This could result in waste of analysts' time through the necessity of further confirmatory testing or the rejection of evidence due to it being falsely believed a non-body-fluid stain with no relevance to the investigation. And at the time of this writing, there is no confirmatory test for the presence of saliva (3, 4).

One way to develop a confirmatory method of body fluid identification would be to look for molecular species that are unique to a particular body fluid. While DNA can provide individually-identifiable information for evidence, it is almost universally present in tissues and cannot provide information on the body fluid source of a stain. RNA, on the other hand, is generally present in much higher concentrations than DNA and has variability based on tissue identity. Additionally, RNA can be co-isolated with DNA, reducing consumption of the evidence and potentially increasing the speed of the workflow (9, 10). Using a confirmatory technique that relies on nucleic acids avoids the risk of presumptive techniques that would degrade the sample, saves valuable analyst time, and helps increase the rate at which a case can be processed.

Thus, the goal of identifying a stain could be achieved through the use of RNA species that are only expressed in a given body fluid, or have alternatively-spliced variants that are processed differently for a given body fluid. Tissue-specific RNA species have been used previously for body fluid identification (11-13). The caveat to these past techniques is that they rely on polymerase chain reaction (PCR) for identification of the tissue-specific RNA species. PCR is highly useful and accurate in the identification of body fluids, but it also requires specialized equipment and training, and is a time-consuming process.

Another method of identifying these tissue-specific RNA species would be to use a particular kind of probe that only binds and fluoresces in the presence of a target RNA species. Molecular Beacons (MB) are a form of nanotechnology that can function as this type of probe. MBs are short sequences of DNA or RNA designed to bind to a target sequence and that have a quencher or fluorophore on either end and small flanking sequences that result in self-folding into a hairpin shape (Figure 1; 14). These MBs are stable in this hairpin shape and do not fluoresce, but if mixed with the target RNA they will unfold, bind the target, and produce a positive signal in the form of fluorescence. If, however, the molecular beacon is mixed with a non-target RNA, it stays in the hairpin shape and reveals no increased fluorescence.

Specifically, the proposed MBs use a quantum dot (QD) fluorophore. QDs were chosen because they have a much higher fluorescence than traditional organic fluorophores, and are highly stable as well (15, 16). The specific quantum dots used have a CdSe core with a ZnS coating. The wavelength of fluorescence is based on the size of the CdSe core, and the intensity of the fluorescence increases with the wavelength (Table 1; 17). This product would be known as a quantum dot molecular beacon (QDMB), to be distinguished from traditional MBs; the theoretical structure is given in Figure 2.

This reaction between the QDMB and the target RNA occurs at room temperature in a short amount of time and requires only a small fluorospectrometer and a laptop for determination of the response. If combined with a fast spin-column extraction technique, analysis of potential body fluids resulting in a confirmatory response can occur in as little as 30 minutes. Such a technique would also require only a minimum of training compared to PCR-based analysis. As such, it is believed this holds great potential for increasing the value of evidence retrieved by ensuring that evidence collected and returned to the crime laboratory is of actual scientific value.

This evidentiary value can be increased further if greater information is able to be retrieved from the sample using the same technique. It has been established that tissue-specific RNA species can be used for body fluid identification, but there may be more information that can be derived from the RNA in the stains. For instance, if the sex of the ‘donor’ could be determined at the scene, this may provide an investigative lead and will give more information to direct the continued processing of the evidence. This method also has the potential for detecting mixtures of fluids, which can affect the later processing as well.

The main objective of this thesis is to refine past research upon QDMBs for body fluid identification and to expand their usefulness as a forensic analytical technique. A rapid test for body fluid identification and determination of sex of the person that deposited the stain may be a great boon to investigators by helping narrow down which evidence may be of the highest value to the investigation. The preliminary detection limitations of this technique were also tested, through a dilution series and the aging of samples.

Figure 1: A traditional molecular beacon and its method of function. Upon ‘discovery’ of its target sequence, it is more stable to unfold from the hairpin and bind to the target, resulting in fluorescence. GeneLink, 2015.

Figure 2: Theoretical structure of QDMB. Note the multiple quenchers attached to the QD fluorophore. The red box represents biotin and the green shapes are the streptavidin, which are responsible for the conjugation. Courtesy of Dr. Stephanie Young.

Table 1: Fluorescence values for organic fluorophores versus quantum dots.

Fluorophore	Emission Wavelength (nm)	Extinction Coefficient (M ⁻¹ cm ⁻¹)
FAM ^[18]	525	74,850
Texas Red ^[18]	583	136,000
QD525 ^[19]	525	710,000
QD800 ^[19]	800	12,600,000

Methods

Analysis of Tissue-Specific RNA

The development of this methodology began with the identification of tissue-specific RNAs. An extensive review of the literature was undertaken to choose candidate genes that display tissue-specific expression or tissue-specific alternative splice variants. After selection of candidate genes, PCR primers were designed to confirm the tissue-specificity of the RNA. If an mRNA was found to not be tissue specific, it was rejected, and the literature search continued. This was repeated until a tissue-specific RNA was found for each body fluid or subject of interest (Table 2).

The blood, saliva, and human-specific beacons were developed as part of past research in the Bishop lab (26). The rationale for their selection was based on published data and the citations are included in Table 2. The male-specific beacon was originally believed to be semen-specific, but further testing showed that the gene variant selected, originally thought to be a splice variant only found in testes, is also found in male blood. XIST was chosen as the female-specific beacon due to its role in the X-inactivation process and recent research showing that it is significantly upregulated in the female blood transcriptome (24, 25).

Development of Molecular Beacons

Once target RNAs were chosen, molecular beacons were designed based on those RNAs. The MBs were designed to target exon junctions so that the beacons would not bind DNA instead and thus no longer be tissue-specific. Lengths of 20-25 nt were chosen, similar to PCR primers, as this length provides specificity and stability to the target-beacon complex. The short flanking sequences were then added to allow self-folding, but they are sufficiently short such that it is more thermodynamically stable for the target-beacon complex to form when the target RNA

is present. Molecular beacons were synthesized according to established protocols for streptavidin-biotin-linked MBs with few modifications (27).

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, and these sequences can be found in Table 3. Six nucleotides of complementary sequence were designed to flank either end of the oligonucleotide to create the stem portion of the MB. Streptavidin-coated QDs were obtained from Life Technologies (Carlsbad, CA). The RNA species, the emission wavelengths of the QD label, and the corresponding quencher molecule used for each body fluid can be found in Table 3.

Conjugation of Molecular Beacons to Quantum Dots

As the molecular beacon oligos were received from a different source than the quantum dot fluorophores, they needed to be conjugated in the lab. This conjugation technique was a modification of the technique used by Cady (27). Twenty-five pmol of the chosen QD, 1 mmol of the MB oligo, and QD incubation buffer to a final volume of 400 μ L were combined in a sterile 1.5 mL tube. This mixture was incubated at room temperature for one hour. A Millipore Amicon 50K spin-column membrane was wet by adding 100 μ L of QD buffer and running in a centrifuge at 13,000 xg for 1 minute. The flow-through of this step was discarded. Once the incubation was complete, the 400 μ L QD/oligo mixture was added to the Millipore spin column, and centrifuged at 13,000 xg for 5 minutes. This flow-through was reserved for fluorescence analysis. The flow-through was analyzed on the fluorospectrometer to test for any fluorescence which would indicate loss of QDs in the conjugation process; if no fluorescence is seen then the

conjugation is highly efficient. The spin column was washed twice using 400 μL of the buffer, and centrifuged at 13,000 $\times g$ for 5 minutes each time. The spin column was placed upside-down in a collection tube, and centrifuged at 1000 g for 2 minutes to collect the QDMBs. A significant reduction of fluorescence was observed between the QD and the conjugated QDMB, as the conjugation was successful.

Analysis of Sample Using QDMBs

For analysis of a sample, 20 μL of RNA was combined with 2 μL of the QDMB solution and incubated for 10 minutes. This mixture was analyzed by pipetting 2 μL onto the pedestal of the NanoDrop 3300 (ThermoScientific). The blank for analysis was 2 μL nuclease-free water, the background fluorescence was established using 2 μL QDMB in 20 μL water, and the positive control was 2 μL of unquenched QD in 20 μL water.

Specificity of QDMBs

Each QDMB was tested for specificity by combining QDMBs with fresh RNA extracted from blood, semen, and saliva. Extractions were performed using the manufacturer's protocol for TRI-Reagent BD, using 30 μL of the given body fluid (MRC, Inc., Cincinnati, OH). Once extracted, the RNA was analyzed using the protocol given above.

Aging of Samples

As the initial step in determining limits of detection for this technique, samples of each body fluid (blood, semen, and saliva) were deposited upon fabric, allowed to dry, and aged for a predetermined time period in the lab under ambient conditions. Samples were aged for 0, 1, 3, 7, or 14 days, then RNA was extracted and tested as described previously.

Statistical Analysis

Results of each step were analyzed using one-way ANOVA followed by Tukey-Kramer HSD, to test whether that there was a significant difference between positive and negative results with each beacon. ANOVA including Dunnet's test was performed for the data of the aging study, as this will show the significance relative to a control.

Table 2: RNA identities and primer sequences for body fluid identification.

Body Fluid	RNA	RNA Alternatively Spliced?	Primer Sequence
Blood	4.1R ^[20]	Yes	FP: 5'- AGCATGGAAGAAAAAGAGAGAAAGA -3' RP: 5'- TCTTCACCAGGGGAGGTCCT -3'
Saliva	EMP1 ^[21]	No	FP: 5'- ACCCTTCAGAACTCTCTTTGCTCA -3' RP: 5'- AACGCCGATGATGAAGCTG -3'
Semen	SG ^[22]	No	FP: 5'- ATGGGACAAAAAGGTGGATCA -3' RP: 5'- GACTTTTTTCGGGACTGGTCA -3'
Male-specific	hHK1 ^[23]	Yes	FP: 5'- GCAAGGACAGTGC GTTCAAGA -3' RP: 5'- CCGCATGGCATAGAGATACTTG -3'
Female-specific	XIST ^[24, 25]	No	FP: 5'- CTCTTTAGAATACTGATCCCAT -3' RP: 5'- CTCCACCTAGGGATCGTCAA -3'
Blood/Saliva/Semen (Human-Specific)	β -actin ^[26]	No	FP: 5'- CCATCCATGTACGTTGCTATCCAGGCTGT -3' RP: 5'- GCAGCCGTGGCCATCTC -3'

Table 3: Body fluid specific RNA identities with QD wavelength, quencher, and beacon sequence. The underlined portion represents the self-complementary stem that leads to the formation of the molecular beacon hairpin structure.

RNA	QD	Iowa Black Quencher	MB Oligonucleotide Sequence
4.1R	585 nm	FQ	5'- <u>CCCGGA</u> UCUUUCUCUCUUUUUCUCCAUGCU <u>UCCGGG</u> -3'
EMP1	605 nm	RQ	5'- <u>CCCGGA</u> UGAGCAAAGAGAGUUCUGAAGGGU <u>UCCGGG</u> -3'
SG	585 nm	FQ	5'- <u>CCCGGA</u> UUGGGACAAAAAGGUGGAUCA <u>UCCGGG</u> -3'
hHK1	655 nm	RQ	5'- <u>CCCGGA</u> UCUUGAACGCACUGUCCUUGCU <u>UCCGGG</u> -3'
XIST	625 nm	RQ	5'- <u>CCCGGA</u> CUCUUUAGAAUACUGAUCCCAUU <u>UCCGGG</u> -3'
β -actin	565 nm	FQ	5'- <u>CCCGGA</u> ACAGCCUGGAUAGCAACGUACAUGGCUGG <u>UCCGGG</u> -3'

Results

Effective Conjugation of MB to QD

The attachment of the biotinylated MB containing an Iowa Black quencher molecule to the streptavidin-coated QD resulted in a reduction of fluorescence. Effective conjugation is demonstrated in Figure 3, where a reduction in fluorescence of the QDMB is demonstrated relative to the QD alone. The semen QDMB showed the greatest level of quenching, with a 93% reduction in fluorescence. The blood QDMB showed the lowest level of quenching, with a reduction in fluorescence of 55%. The saliva and human-specific QDMBs showed a 64% and 66% reduction in fluorescence, respectively. The female QDMB showed 87% quenching, while the male QDMB displayed 82% quenching effectiveness.

Tissue Specificity of Conjugated QDMBs

The QDMBs only showed a significant increase in fluorescence when combined with their appropriate target RNA and did not show a significant increase when combined with non-target RNA species (Fig. 4). This demonstration of increasing fluorescence when only in contact with target RNA species can also be seen in Figure 5, where error bars are included. It should be noted that the sex-specific beacons did not identify saliva. Saliva is not separated by sex because the sex-specific beacons do not give a response to saliva. For this study, n=14 saliva donors, 7 female blood donors, 7 male blood donors, and 4 semen donors.

An ANOVA analysis with Tukey-Kramer HSD was also performed on the data, to provide statistical support for the conclusions observed. Each beacon tested resulted in a p-value of <0.0001 in the ANOVA for a positive response, when compared to non-target background

fluorescence. The analytical results of the statistical analysis for the female-specific beacon are provided as an example of the output (Figure 6). The other analytical outputs can be found in Appendix 1.

Effectiveness Using Aged Samples

Results were mixed for the testing of effectiveness with aged samples. Blood and saliva were only detectable for 3 days, based on the design of the study. Semen was detectable for 7 days. The male and female beacons were both detectable in blood at 14 days; they may persist longer but the study only contained samples for 14 days of testing. It is of note that the male beacon displayed an upward trend in the fluorescence level with the length of time of aging of the sample. This may be coincidence, or an effect of the aging process. It should also be noted that the male and female beacons were used to test aged blood samples, and not stains of other fluids. For this study, $n=3$ samples from one donor per fluid per day.

The statistical analysis of the aged samples supports the conclusions reached from the graphing. The female-specific and male-specific beacons displayed a p-value of <0.0001 for each aging period, when compared to the control in ANOVA. The blood-specific beacon displayed a p-value of <0.0001 for days 0, 1, and 3, while days 7 and 14 were not significantly different from the control, meaning a negative response. The semen-specific beacon displayed a p-value of <0.0001 for days 0, 1, 3, and 7, while day 14 was not significantly different from the control. The saliva-specific beacon displayed a p-value of <0.0001 for days 0, 1, 3, and 7, while day 14 was not significantly different from the control. The caveat to this is that while day 7 was significantly different from the control, it was actually due to lower RFU readings, still meaning a negative response. The full results can be found in Appendix 1.

Figure 3: Relative fluorescence of QD versus QDMB. The relative fluorescence of each QD (in black) is compared to that of the QD when conjugated to form the QDMB. A: Blood, B: Saliva, C: Semen, D: Female, E: Male, F: Human.

Figure 4: Tissue specificity testing of QDMBs. These graphs demonstrate that the QDMBs only give fluorescence increases above background when in contact with RNA from the target body fluid. A: Blood, B: Saliva, C: Semen, D: Female, E: Male, F: Human. N=14 saliva donors, 7 female blood donors, 7 male blood donors, 4 semen donors.

Figure 5: A comparison of target and non-target QDMB fluorescence expression. The QDMB displays a significant increase in fluorescence only when combined with the target RNA, and shows little or no increase relative to background when combined with non-target RNA. N=14 saliva donors, 7 female blood donors, 7 male blood donors, 4 semen donors. $P < 0.0001$ for each positive result, as determined by ANOVA (See Figure 7 and Appendix).

Figure 6: Results of aging of samples in days, tested with tissue-specific beacon. N= 3 samples from one donor per fluid per day. P-values reported in text.

Figure 7: The JMP output for ANOVA analysis of the female-specific beacon testing data.

Discussion

The purpose of this study was to continue development of a novel, confirmatory method of body fluid identification. The development of such a technique would reduce the amount of evidence retrieved while increasing the quality, and thus ease the workflow for evidence analysis in crime laboratories. 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. This study also shows that the sex of the person can be determined from a bloodstain, without the use of PCR or other time-consuming techniques (28, 29). The presented method, especially once optimized in multiplex, may help to reduce the amount of backlog that is currently plaguing crime laboratories nationwide.

The MB oligonucleotides were linked to their QDs using biotin-streptavidin interactions. The QDMBs 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 QDMBs was more highly quenched than others, exhibiting a range of 97.4% to 48.3% reduction in fluorescence. As the QDMBs are not all equally quenched, there is room for further optimization. This variation is likely the result of natural differences in the fluorescence levels of the QDs, and the number of quenching MB probes attached to each QD (Figure 2). Each wavelength of QD has a different fluorescence intensity, which can be a challenge in quenching. Further refinement of the attachment chemistry to increase the quenching of the QDs will lead to an increase in the fluorescence above background detected when the QDMB meets its target RNA.

Refinement of the assay may also increase the viability of multiplexing of the reactions. Understanding the functioning of the probes in singleplex is useful; however, a multiplex of reactions will shorten the amount of time necessary for sample analysis and reduce the amount of RNA necessary for analysis. There is also room for improvement on the basis of the binding of the QDMB to the target RNA. While RNA is generally visualized as a line, such as in Figure 1, in reality it tends to have secondary structure complicating this binding process. One possibility would be to heat the RNA and break hydrogen bonds holding the secondary structure; however, some preliminary work shows that heating the QDMB denatures the streptavidin-biotin linkage and eliminates the effectiveness of the QDMB.

Once optimized, there is room for further development and experimentation. Further study of the effect of aging of samples on the results could be performed to determine if there are reliable trends, and to determine what is causing the early drop-out of certain RNA species. If the trends are consistent across longer and larger studies, however, the results of the test could provide a rough estimate of the time of deposit of the stain. Past research using PCR-based techniques have shown that RNA can be detected in body fluid stains after longer periods of aging and can be used to determine the age of the stain (30, 31). Considering that the RNAs are known to exist for longer periods of time and are detectable using PCR, there may be some factor in the aging that affects the binding and fluorescence of the QDMB specifically. Also, there is the potential for the development of more QDMBs that could be incorporated into the technique to identify other tissue-specific RNA species of interest.

There are caveats to the sex-specificity beacons that can be controlled. Primarily, these are chromosomal disorders such as Turner's and Klinefelter's Syndromes. Turner's is an XO genotype, meaning a phenotypic female with a single X chromosome, and occurs in 1 in 2500

live births worldwide (32). A Turner female would not express XIST and thus would not test positive for our female-specific beacon; however, they would not test positive for the male-specific, either. A bloodstain that does not test positive for either sex-specific beacon could be logically surmised to be from a female with Turner's syndrome. Klinefelter's syndrome is a phenotypic male with multiple X chromosomes in addition to a Y chromosome, and occurs in 1 in 500 to 1000 live male births (33). A person with Klinefelter's would theoretically test positive for both sex-specific beacons and lead to the conclusion it is a mixed sample; later STR testing would confirm that it is in fact a single male donor.

We believe this technique offers multiple advantages in comparison to current presumptive and confirmatory tests for body fluid identification. Our technique can be used to process multiple samples in less than hour and does not require PCR. Without the requirement for PCR, the equipment, cost, and training required for the technique are all reduced. The cost of a multiplex reaction, combining the three body fluids with a human-specific beacon, would be approximately \$10 per sample for this technique. Including the sex-specific beacons would only be an increase of \$1-2 per sample. The technique provides a confirmatory answer to the identity of a body fluid or mixture of fluids, compared to at least two separate lab tests required to determine this currently. This may also be the first confirmatory technique for saliva that does not rely upon PCR. In addition, the technique can identify the sex of the depositor of a blood stain, which currently requires PCR analysis. This technique also does not degrade the DNA of a sample, as will some presumptive tests (8). Overall, this technique has the potential to be a highly useful tool in the reduction of the national DNA backlog and improving the value of RNA in forensic science.

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Appendix 1: JMP Output

Semen-specific Test

Oneway Analysis of RFU By Treatment

Oneway Anova

Summary of Fit

Rsquare 0.99412
Adj Rsquare 0.993336
Root Mean Square Error 699.115
Mean of Response 29828.23
Observations (or Sum Wgts) 35

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	4	2479087134	619771783	1268.045	<.0001*
Error	30	14662852.3	488761.74		
C. Total	34	2493749986			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Blank	3	24058.3	403.63	23234	24883
Blood (Female)	7	25427.4	264.24	24888	25967
Blood (Male)	7	25738.6	264.24	25199	26278
Saliva	14	28747.4	186.85	28366	29129
Semen	4	52796.8	349.56	52083	53511

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

q* Alpha
2.90061 0.05

LSD Threshold Matrix

Abs(Dif)-HSD	Semen	Saliva	Blood (Male)	Blood (Female)	Blank
Semen	-1434	22900	25787	26098	27190
Saliva	22900	-766	2070	2381	3399
Blood (Male)	25787	2070	-1084	-773	281
Blood (Female)	26098	2381	-773	-1084	-30
Blank	27190	3399	281	-30	-1656

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level	Mean
Semen	52796.750
Saliva	28747.429
Blood (Male)	25738.571
Blood (Female)	25427.429
Blank	24058.333

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Semen	Blank	28738.42	533.9579	27189.6	30287.22	<.0001*
Semen	Blood (Female)	27369.32	438.1935	26098.3	28640.35	<.0001*
Semen	Blood (Male)	27058.18	438.1935	25787.1	28329.21	<.0001*
Semen	Saliva	24049.32	396.3609	22899.6	25199.01	<.0001*
Saliva	Blank	4689.10	444.7832	3399.0	5979.24	<.0001*
Saliva	Blood (Female)	3320.00	323.6273	2381.3	4258.72	<.0001*
Saliva	Blood (Male)	3008.86	323.6273	2070.1	3947.57	<.0001*
Blood (Male)	Blank	1680.24	482.4352	280.9	3079.59	0.0124*
Blood (Female)	Blank	1369.10	482.4352	-30.3	2768.45	0.0575
Blood (Male)	Blood (Female)	311.14	373.6927	-772.8	1395.08	0.9183

Comparisons with a control using Dunnett's Method

Control Group = Blank

Confidence Quantile

Idl Alpha
2.50127 0.05

LSD Threshold Matrix

Level	LSD	p-Value
Semen	27403	<.0001*
Saliva	3577	<.0001*
Blood (Male)	473.5	0.0048*
Blood (Female)	162.4	0.0234*
Blank	-1428	1.0000

Positive values show pairs of means that are significantly different.

Saliva-specific Test

Oneway Anova

Summary of Fit

Rsquare 0.977593
Adj Rsquare 0.974605
Root Mean Square Error 1585.245
Mean of Response 50273.49
Observations (or Sum Wgts) 35

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	4	3289144048	822286012	327.2128	<.0001*
Error	30	75390015.1	2513000.5		
C. Total	34	3364534063			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Blank	3	42288.0	915.24	40419	44157
Blood (Female)	7	42089.6	599.17	40866	43313
Blood (Male)	7	40954.4	599.17	39731	42178
Saliva	14	62038.9	423.67	61174	62904
Semen	4	45714.0	792.62	44095	47333

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

q* Alpha
2.90061 0.05

LSD Threshold Matrix

Abs(Dif)-HSD	Saliva	Semen	Blank	Blood (Female)	Blood (Male)
Saliva	-1738	13718	16825	17821	18956
Semen	13718	-3251	-86	742	1878
Blank	16825	-86	-3754	-2975	-1839
Blood (Female)	17821	742	-2975	-2458	-1323
Blood (Male)	18956	1878	-1839	-1323	-2458

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level	Mean
Saliva	62038.857
Semen	45714.000
Blank	42288.000
Blood (Female)	42089.571
Blood (Male)	40954.429

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Saliva	Blood (Male)	21084.43	733.826	18955.9	23212.97	<.0001*
Saliva	Blood (Female)	19949.29	733.826	17820.7	22077.83	<.0001*
Saliva	Blank	19750.86	1008.547	16825.5	22676.26	<.0001*
Saliva	Semen	16324.86	898.749	13717.9	18931.78	<.0001*
Semen	Blood (Male)	4759.57	993.605	1877.5	7641.63	0.0004*
Semen	Blood (Female)	3624.43	993.605	742.4	6506.49	0.0082*
Semen	Blank	3426.00	1210.751	-85.9	6937.92	0.0586
Blank	Blood (Male)	1333.57	1093.923	-1839.5	4506.62	0.7405
Blood (Female)	Blood (Male)	1135.14	847.349	-1322.7	3592.97	0.6693
Blank	Blood (Female)	198.43	1093.923	-2974.6	3371.47	0.9997

Comparisons with a control using Dunnett's Method

Control Group = Blank

Confidence Quantile

Idl Alpha
2.50127 0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
Saliva	17228	<.0001*
Semen	397.6	0.0239*
Blank	-3238	1.0000
Blood (Female)	-2538	0.9984
Blood (Male)	-1403	0.4871

Positive values show pairs of means that are significantly different.

Blood-specific Test

Oneway Analysis of RFU By Treatment

Oneway Anova

Summary of Fit

Rsquare 0.986601
Adj Rsquare 0.984814
Root Mean Square Error 1309.828
Mean of Response 13326.49
Observations (or Sum Wgts) 35

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	4	3789815768	947453942	552.2423	<.0001*
Error	30	51469473.2	1715649.1		
C. Total	34	3841285241			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Blank	3	5843.0	756.23	4299	7387
Blood (Female)	7	26428.7	495.07	25418	27440
Blood (Male)	7	25694.9	495.07	24684	26706
Saliva	14	4670.1	350.07	3955	5385
Semen	4	4663.0	654.91	3325	6001

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

q*	Alpha
2.90061	0.05

LSD Threshold Matrix

Abs(Dif)-HSD

	Blood (Female)	Blood (Male)	Blank	Saliva	Semen
Blood (Female)	-2031	-1297	17964	20000	19384
Blood (Male)	-1297	-2031	17230	19266	18651
Blank	17964	17230	-3102	-1244	-1722
Saliva	20000	19266	-1244	-1436	-2147
Semen	19384	18651	-1722	-2147	-2687

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level	Mean
Blood (Female) A	26428.714
Blood (Male) A	25694.857
Blank B	5843.000
Saliva B	4670.071
Semen B	4663.000

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Blood (Female)	Semen	21765.71	820.978	19384.4	24147.05	<.0001*
Blood (Female)	Saliva	21758.64	606.332	19999.9	23517.38	<.0001*
Blood (Male)	Semen	21031.86	820.978	18650.5	23413.19	<.0001*
Blood (Male)	Saliva	21024.79	606.332	19266.1	22783.52	<.0001*
Blood (Female)	Blank	20585.71	903.867	17963.9	23207.48	<.0001*
Blood (Male)	Blank	19851.86	903.867	17230.1	22473.62	<.0001*
Blank	Semen	1180.00	1000.398	-1721.8	4081.76	0.7627
Blank	Saliva	1172.93	833.324	-1244.2	3590.08	0.6277
Blood (Female)	Blood (Male)	733.86	700.132	-1297.0	2764.67	0.8309
Saliva	Semen	7.07	742.603	-2146.9	2161.07	1.0000

Comparisons with a control using Dunnett's Method

Control Group = Blank

Confidence Quantile

Id1	Alpha
2.50127	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
Blood (Female)	18325	<.0001*
Blood (Male)	17591	<.0001*
Blank	-2675	1.0000
Saliva	-911	0.3747
Semen	-1322	0.5127

Positive values show pairs of means that are significantly different.

Male-specific Test

Oneway Anova

Summary of Fit

Rsquare 0.95957
Adj Rsquare 0.95418
Root Mean Square Error 3190.851
Mean of Response 60469.54
Observations (or Sum Wgts) 35

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	4	7249541361	1.8124e+9	178.0072	<.0001*
Error	30	305445850	10181528		
C. Total	34	7554987211			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Blank	3	52028.0	1842.2	48266	55790
Blood (Female)	7	52185.0	1206.0	49722	54648
Blood (Male)	7	81242.9	1206.0	78780	83706
Saliva	14	49759.9	852.8	48018	51502
Semen	4	82429.0	1595.4	79171	85687

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

q* Alpha
2.90061 0.05

LSD Threshold Matrix

Abs(Dif)-HSD	Semen	Blood (Male)	Blood (Female)	Blank	Saliva
Semen		-6545	-4615	24443	23332
Blood (Male)	-6545		-4947	24111	22828
Blood (Female)	24443	24111		-4947	-6230
Blank	23332	22828	-6230		-7557
Saliva	27422	27199	-1859	-3620	

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level	Mean
Semen	A
Blood (Male)	A
Blood (Female)	B
Blank	B
Saliva	B

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Semen	Saliva	32669.07	1809.042	27421.7	37916.40	<.0001*
Blood (Male)	Saliva	31482.93	1477.077	27198.5	35767.35	<.0001*
Semen	Blank	30401.00	2437.052	23332.1	37469.94	<.0001*
Semen	Blood (Female)	30244.00	1999.972	24442.9	36045.14	<.0001*
Blood (Male)	Blank	29214.86	2201.896	22828.0	35601.70	<.0001*
Blood (Male)	Blood (Female)	29057.86	1705.581	24110.6	34005.08	<.0001*
Blood (Female)	Saliva	2425.07	1477.077	-1859.4	6709.50	0.4836
Blank	Saliva	2268.07	2030.048	-3620.3	8156.45	0.7962
Semen	Blood (Male)	1186.14	1999.972	-4615.0	6987.28	0.9751
Blood (Female)	Blank	157.00	2201.896	-6229.8	6543.84	1.0000

Comparisons with a control using Dunnett's Method

Control Group = Blank

Confidence Quantile

Idl Alpha
2.50127 0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
Semen	24305	<.0001*
Blood (Male)	23707	<.0001*
Blood (Female)	-5351	1.0000
Blank	-6517	1.0000
Saliva	-2810	0.5542

Positive values show pairs of means that are significantly different.

Female-Specific Test

Oneway Anova

Summary of Fit

Rsquare 0.968504
Adj Rsquare 0.964305
Root Mean Square Error 2468.651
Mean of Response 36732.69
Observations (or Sum Wgts) 35

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	4	5621941040	1.4055e+9	230.6253	<.0001*
Error	30	182827085	6094236.2		
C. Total	34	5804768126			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Blank	3	29775.7	1425.3	26865	32686
Blood (Female)	7	62072.9	933.1	60167	63978
Blood (Male)	7	30090.0	933.1	28184	31996
Saliva	14	30520.7	659.8	29173	31868
Semen	4	30971.8	1234.3	28451	33493

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

q* Alpha
2.90061 0.05

LSD Threshold Matrix

Abs(Dif)-HSD

	Blood (Female)	Semen	Saliva	Blood (Male)	Blank
Blood (Female)	-3827	26613	28237	28155	27356
Semen	26613	-5063	-3609	-3606	-4273
Saliva	28237	-3609	-2706	-2884	-3811
Blood (Male)	28155	-3606	-2884	-3827	-4627
Blank	27356	-4273	-3811	-4627	-5847

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level	Mean
Blood (Female) A	62072.857
Semen B	30971.750
Saliva B	30520.714
Blood (Male) B	30090.000
Blank B	29775.667

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Blood (Female)	Blank	32297.19	1703.531	27355.9	37238.47	<.0001*
Blood (Female)	Blood (Male)	31982.86	1319.549	28155.4	35810.36	<.0001*
Blood (Female)	Saliva	31552.14	1142.763	28237.4	34866.85	<.0001*
Blood (Female)	Semen	31101.11	1547.309	26613.0	35589.25	<.0001*
Semen	Blank	1196.08	1885.463	-4272.9	6665.08	0.9682
Semen	Blood (Male)	881.75	1547.309	-3606.4	5369.89	0.9785
Saliva	Blank	745.05	1570.578	-3810.6	5300.68	0.9891
Semen	Saliva	451.04	1399.593	-3608.6	4510.71	0.9975
Saliva	Blood (Male)	430.71	1142.763	-2884.0	3745.43	0.9955
Blood (Male)	Blank	314.33	1703.531	-4626.9	5255.61	0.9997

Comparisons with a control using Dunnett's Method

Control Group = Blank

Confidence Quantile

Idl Alpha
2.50127 0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
Blood (Female)	28036	<.0001*
Semen	-3520	0.8764
Saliva	-3183	0.9488
Blood (Male)	-3947	0.9983
Blank	-5042	1.0000

Positive values show pairs of means that are significantly different.

Human-Specific Test

Oneway Analysis of RFU By Treatment

Oneway Anova

Summary of Fit

Rsquare 0.969054
 Adj Rsquare 0.964928
 Root Mean Square Error 141.7188
 Mean of Response 6489.143
 Observations (or Sum Wgts) 35

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	4	18867576	4716894	234.8557	<.0001*
Error	30	602527	20084		
C. Total	34	19470102			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Blank	3	4172.67	81.821	4005.6	4339.8
Blood (Female)	7	6723.43	53.565	6614.0	6832.8
Blood (Male)	7	6807.00	53.565	6697.6	6916.4
Saliva	14	6795.57	37.876	6718.2	6872.9
Semen	4	6187.75	70.859	6043.0	6332.5

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

q* 2.90061
 Alpha 0.05

LSD Threshold Matrix

Abs(Dif)-HSD	Blood (Male)	Saliva	Blood (Female)	Semen	Blank
Blood (Male)	-219.7	-178.9	-136.2	361.6	2350.7
Saliva	-178.9	-155.4	-118.1	374.8	2361.4
Blood (Female)	-136.2	-118.1	-219.7	278.0	2267.1
Semen	361.6	374.8	278.0	-290.7	1701.1
Blank	2350.7	2361.4	2267.1	1701.1	-335.6

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level	Mean
Blood (Male) A	6807.0000
Saliva A	6795.5714
Blood (Female) A	6723.4286
Semen B	6187.7500
Blank C	4172.6667

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Blood (Male)	Blank	2634.333	97.7953	2350.67	2917.999	<.0001*
Saliva	Blank	2622.905	90.1628	2361.38	2884.432	<.0001*
Blood (Female)	Blank	2550.762	97.7953	2267.10	2834.428	<.0001*
Semen	Blank	2015.083	108.2395	1701.12	2329.044	<.0001*
Blood (Male)	Semen	619.250	88.8270	361.60	876.902	<.0001*
Saliva	Semen	607.821	80.3470	374.77	840.877	<.0001*
Blood (Female)	Semen	535.679	88.8270	278.03	793.331	<.0001*
Blood (Male)	Blood (Female)	83.571	75.7519	-136.16	303.298	0.8035
Saliva	Blood (Female)	72.143	65.6031	-118.15	262.432	0.8053
Blood (Male)	Saliva	11.429	65.6031	-178.86	201.717	0.9998

Comparisons with a control using Dunnett's Method

Control Group = Blank

Confidence Quantile

Idl 2.50127
 Alpha 0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
Blood (Male)	2390	<.0001*
Saliva	2397	<.0001*
Blood (Female)	2306	<.0001*
Semen	1744	<.0001*
Blank	-289	1.0000

Positive values show pairs of means that are significantly different.

Aged Semen Test

Oneway Anova

Summary of Fit

Rsquare 0.998603
Adj Rsquare 0.998021
Root Mean Square Error 505.7469
Mean of Response 37362.22
Observations (or Sum Wgts) 18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	5	2194238242	438847648	1715.724	<.0001*
Error	12	3069358.67	255779.89		
C. Total	17	2197307601			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Blank	3	23811.7	291.99	23175	24448
Day 0	3	37176.0	291.99	36540	37812
Day 1	3	40840.7	291.99	40204	41477
Day 3	3	51915.3	291.99	51279	52552
Day 7	3	47757.7	291.99	47121	48394
Day 14	3	22672.0	291.99	22036	23308

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

q* Alpha
3.35886 0.05

LSD Threshold Matrix

Abs(Dif)-HSD

	Day 3	Day 7	Day 1	Day 0	Blank	Day 14
Day 3	-1387	2771	9688	13352	26717	27856
Day 7	2771	-1387	5530	9195	22559	23699
Day 1	9688	5530	-1387	2278	15642	16782
Day 0	13352	9195	2278	-1387	11977	13117
Blank	26717	22559	15642	11977	-1387	-247
Day 14	27856	23699	16782	13117	-247	-1387

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level	Mean
Day 3 A	51915.333
Day 7 B	47757.667
Day 1 C	40840.667
Day 0 D	37176.000
Blank E	23811.667
Day 14 E	22672.000

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Day 3	Day 14	29243.33	412.9406	27856.3	30630.34	<.0001*
Day 3	Blank	28103.67	412.9406	26716.7	29490.68	<.0001*
Day 7	Day 14	25085.67	412.9406	23698.7	26472.68	<.0001*
Day 7	Blank	23946.00	412.9406	22559.0	25333.01	<.0001*
Day 1	Day 14	18168.67	412.9406	16781.7	19555.68	<.0001*
Day 1	Blank	17029.00	412.9406	15642.0	18416.01	<.0001*
Day 3	Day 0	14739.33	412.9406	13352.3	16126.34	<.0001*
Day 0	Day 14	14504.00	412.9406	13117.0	15891.01	<.0001*
Day 0	Blank	13364.33	412.9406	11977.3	14751.34	<.0001*
Day 3	Day 1	11074.67	412.9406	9687.7	12461.68	<.0001*
Day 7	Day 0	10581.67	412.9406	9194.7	11968.68	<.0001*
Day 7	Day 1	6917.00	412.9406	5530.0	8304.01	<.0001*
Day 3	Day 7	4157.67	412.9406	2770.7	5544.68	<.0001*
Day 1	Day 0	3664.67	412.9406	2277.7	5051.68	<.0001*
Blank	Day 14	1139.67	412.9406	-247.3	2526.68	0.1331

Comparisons with a control using Dunnett's Method

Control Group = Blank

Confidence Quantile

Idl Alpha
2.90130 0.05

LSD Threshold Matrix

Level	LSD	p-Value
Day 3	26906	<.0001*
Day 7	22748	<.0001*
Day 1	15831	<.0001*
Day 0	12166	<.0001*
Blank	-1198	1.0000
Day 14	-58.4	0.0640

Positive values show pairs of means that are significantly different.

Aged Saliva Test

Oneway Analysis of RFU By Treatment

Oneway Anova

Summary of Fit

Rsquare 0.999667
Adj Rsquare 0.999528
Root Mean Square Error 390.3148
Mean of Response 55389.06
Observations (or Sum Wgts) 18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	5	5482611565	1.0965e+9	7197.594	<.0001*
Error	12	1828148	152345.67		
C. Total	17	5484439713			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Blank	3	43054.0	225.35	42563	43545
Day 0	3	53195.3	225.35	52704	53686
Day 1	3	90345.0	225.35	89854	90836
Day 3	3	62892.3	225.35	62401	63383
Day 7	3	40351.7	225.35	39861	40843
Day 14	3	42496.0	225.35	42005	42987

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

q* Alpha
3.35886 0.05

LSD Threshold Matrix

Abs(Dif)-HSD	Day 1	Day 3	Day 0	Blank	Day 14	Day 7
Day 1	-1070	26382	36079	46221	46779	48923
Day 3	26382	-1070	8627	18768	19326	21470
Day 0	36079	8627	-1070	9071	9629	11773
Blank	46221	18768	9071	-1070	-512	1632
Day 14	46779	19326	9629	-512	-1070	1074
Day 7	48923	21470	11773	1632	1074	-1070

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level	Mean
Day 1 A	90345.000
Day 3 B	62892.333
Day 0 C	53195.333
Blank D	43054.000
Day 14 D	42496.000
Day 7 E	40351.667

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Day 1	Day 7	49993.33	318.6907	48922.9	51063.77	<.0001*
Day 1	Day 14	47849.00	318.6907	46778.6	48919.44	<.0001*
Day 1	Blank	47291.00	318.6907	46220.6	48361.44	<.0001*
Day 1	Day 0	37149.67	318.6907	36079.2	38220.11	<.0001*
Day 1	Day 3	27452.67	318.6907	26382.2	28523.11	<.0001*
Day 3	Day 7	22540.67	318.6907	21470.2	23611.11	<.0001*
Day 3	Day 14	20396.33	318.6907	19325.9	21466.77	<.0001*
Day 3	Blank	19838.33	318.6907	18767.9	20908.77	<.0001*
Day 0	Day 7	12843.67	318.6907	11773.2	13914.11	<.0001*
Day 0	Day 14	10699.33	318.6907	9628.9	11769.77	<.0001*
Day 0	Blank	10141.33	318.6907	9070.9	11211.77	<.0001*
Day 3	Day 0	9697.00	318.6907	8626.6	10767.44	<.0001*
Blank	Day 7	2702.33	318.6907	1631.9	3772.77	<.0002*
Day 14	Day 7	2144.33	318.6907	1073.9	3214.77	0.0002*
Blank	Day 14	558.00	318.6907	-512.4	1628.44	0.5274

Comparisons with a control using Dunnett's Method

Control Group = Blank

Confidence Quantile

Idl Alpha
2.90130 0.05

LSD Threshold Matrix

Level	LSD	p-Value
Day 1	46366	<.0001*
Day 3	18914	<.0001*
Day 0	9217	<.0001*
Blank	-925	1.0000
Day 14	-367	0.3268
Day 7	1778	<.0001*

Positive values show pairs of means that are significantly different.

Aged Blood Test

Oneway Anova

Summary of Fit

Rsquare 0.997865
Adj Rsquare 0.996975
Root Mean Square Error 374.4444
Mean of Response 26175.17
Observations (or Sum Wgts) 18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	5	786264863	157252973	1121.564	<.0001*
Error	12	1682503	140208.61		
C. Total	17	787947367			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Blank	3	20801.3	216.19	20330	21272
Day 0	3	30699.0	216.19	30228	31170
Day 1	3	25072.0	216.19	24601	25543
Day 3	3	38661.7	216.19	38191	39133
Day 7	3	21173.0	216.19	20702	21644
Day 14	3	20644.0	216.19	20173	21115

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

q*	Alpha
3.35886	0.05

LSD Threshold Matrix

Abs(Dif)-HSD

	Day 3	Day 0	Day 1	Day 7	Blank	Day 14
Day 3	-1027	6936	12563	16462	16833	16991
Day 0	6936	-1027	4600	8499	8871	9028
Day 1	12563	4600	-1027	2872	3244	3401
Day 7	16462	8499	2872	-1027	-655	-498
Blank	16833	8871	3244	-655	-1027	-870
Day 14	16991	9028	3401	-498	-870	-1027

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level	Mean
Day 3 A	38661.667
Day 0 B	30699.000
Day 1 C	25072.000
Day 7 D	21173.000
Blank D	20801.333
Day 14 D	20644.000

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Day 3	Day 14	18017.67	305.7326	16990.8	19044.58	<.0001*
Day 3	Blank	17860.33	305.7326	16833.4	18887.25	<.0001*
Day 3	Day 7	17498.67	305.7326	16461.8	18515.58	<.0001*
Day 3	Day 1	13589.67	305.7326	12562.8	14616.58	<.0001*
Day 0	Day 14	10055.00	305.7326	9028.1	11081.91	<.0001*
Day 0	Blank	9897.67	305.7326	8870.8	10924.58	<.0001*
Day 0	Day 7	9526.00	305.7326	8499.1	10552.91	<.0001*
Day 3	Day 0	7962.67	305.7326	6935.8	8989.58	<.0001*
Day 0	Day 1	5627.00	305.7326	4600.1	6653.91	<.0001*
Day 1	Day 14	4428.00	305.7326	3401.1	5454.91	<.0001*
Day 1	Blank	4270.67	305.7326	3243.8	5297.58	<.0001*
Day 1	Day 7	3899.00	305.7326	2872.1	4925.91	<.0001*
Day 7	Day 14	528.00	305.7326	-497.9	1555.91	0.5390
Day 7	Blank	371.67	305.7326	-655.2	1398.58	0.8215
Blank	Day 14	157.33	305.7326	-869.6	1184.25	0.9945

Comparisons with a control using Dunnett's Method

Control Group = Blank

Confidence Quantile

Idl	Alpha
2.90130	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
Day 3	16973	<.0001*
Day 0	9011	<.0001*
Day 1	3384	<.0001*
Day 7	-515	0.6364
Blank	-867	1.0000
Day 14	-730	0.9778

Positive values show pairs of means that are significantly different.

Aged Male-specific Test

Oneway Analysis of RFU By Treatment

Oneway Anova

Summary of Fit

Rsquare 0.999774
 Adj Rsquare 0.99968
 Root Mean Square Error 787.7927
 Mean of Response 92437.78
 Observations (or Sum Wgts) 18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	5	3.2982e+10	6.5965e+9	10628.88	<.0001*
Error	12	7447407.33	620617.28		
C. Total	17	3.299e+10			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Blank	3	56308	454.83	55317	57299
Day 0	3	72808	454.83	71817	73799
Day 1	3	74436	454.83	73445	75427
Day 3	3	76989	454.83	75998	77980
Day 7	3	88250	454.83	87259	89241
Day 14	3	185836	454.83	184845	186827

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

q*	Alpha
3.35886	0.05

LSD Threshold Matrix

Abs(Dif)-HSD	Day 14	Day 7	Day 3	Day 1	Day 0	Blank
Day 14	-2161	95425	106686	109239	110867	127367
Day 7	95425	-2161	9100	11653	13281	29781
Day 3	106686	9100	-2161	393	2020	18521
Day 1	109239	11653	393	-2161	-533	15967
Day 0	110867	13281	2020	-533	-2161	14340
Blank	127367	29781	18521	15967	14340	-2161

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level	Mean
Day 14 A	185835.67
Day 7 B	88249.67
Day 3 C	76989.33
Day 1 D	74435.67
Day 0 D	72808.33
Blank E	56308.00

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Day 14	Blank	129527.7	643.2300	127367	131688.2	<.0001*
Day 14	Day 0	113027.3	643.2300	110867	115187.9	<.0001*
Day 14	Day 1	111400.0	643.2300	109239	113560.5	<.0001*
Day 14	Day 3	108846.3	643.2300	106686	111006.9	<.0001*
Day 14	Day 7	97586.0	643.2300	95425	99746.5	<.0001*
Day 7	Blank	31941.7	643.2300	29781	34102.2	<.0001*
Day 7	Day 0	20681.3	643.2300	18521	22841.9	<.0001*
Day 7	Day 1	18127.7	643.2300	15967	20288.2	<.0001*
Day 7	Day 3	16500.3	643.2300	14340	18660.9	<.0001*
Day 7	Day 14	15441.3	643.2300	13281	17601.9	<.0001*
Day 3	Blank	13814.0	643.2300	11653	15974.5	<.0001*
Day 3	Day 0	11260.3	643.2300	9100	13420.9	<.0001*
Day 3	Day 1	4181.0	643.2300	2020	6341.5	0.0003*
Day 3	Day 3	2553.7	643.2300	393	4714.2	0.0178*
Day 1	Blank	1627.3	643.2300	-533	3787.9	0.1898

Comparisons with a control using Dunnett's Method

Control Group = Blank

Confidence Quantile

Idi	Alpha
2.90130	0.05

LSD Threshold Matrix

Level	Abs(Dif)-LSD	p-Value
Day 14	1e+5	<.0001*
Day 7	30075	<.0001*
Day 3	18815	<.0001*
Day 1	16261	<.0001*
Day 0	14634	<.0001*
Blank	-1866	1.0000

Positive values show pairs of means that are significantly different.

Aged Female-specific Test

Oneway Anova

Summary of Fit

Rsquare 0.998257
Adj Rsquare 0.997531
Root Mean Square Error 1221.853
Mean of Response 61264.72
Observations (or Sum Wgts) 18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	5	1.0262e+10	2.0524e+9	1374.783	<.0001*
Error	12	17915098.7	1492924.9		
C. Total	17	1.028e+10			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Blank	3	29433	705.44	27896	30970
Day 0	3	43799	705.44	42262	45336
Day 1	3	66011	705.44	64474	67548
Day 3	3	104292	705.44	102755	105829
Day 7	3	72691	705.44	71154	74228
Day 14	3	51363	705.44	49826	52900

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

q*	Alpha
3.35886	0.05

LSD Threshold Matrix

Abs(Dif)-HSD	Day 3	Day 7	Day 1	Day 14	Day 0	Blank
Day 3	-3351	28250	34930	49578	57141	71508
Day 7	28250	-3351	3329	17977	25540	39907
Day 1	34930	3329	-3351	11297	18861	33227
Day 14	49578	17977	11297	-3351	4212	18579
Day 0	57141	25540	18861	4212	-3351	11015
Blank	71508	39907	33227	18579	11015	-3351

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level	Mean
Day 3 A	104291.67
Day 7 B	72690.67
Day 1 C	66011.00
Day 14 D	51362.67
Day 0 E	43799.33
Blank F	29433.00

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Day 3	Blank	74858.67	997.6388	71507.73	78209.60	<.0001*
Day 3	Day 0	60492.33	997.6388	57141.40	63843.27	<.0001*
Day 3	Day 14	52929.00	997.6388	49578.07	56279.93	<.0001*
Day 7	Blank	43257.67	997.6388	39906.73	46608.60	<.0001*
Day 3	Day 1	38280.67	997.6388	34929.73	41631.60	<.0001*
Day 1	Blank	36578.00	997.6388	33227.07	39928.93	<.0001*
Day 3	Day 7	31601.00	997.6388	28250.07	34951.93	<.0001*
Day 7	Day 0	28891.33	997.6388	25540.40	32242.27	<.0001*
Day 1	Day 0	22211.67	997.6388	18860.73	25562.60	<.0001*
Day 14	Blank	21929.67	997.6388	18578.73	25280.60	<.0001*
Day 7	Day 14	21328.00	997.6388	17977.07	24678.93	<.0001*
Day 1	Day 14	14648.33	997.6388	11297.40	17999.27	<.0001*
Day 0	Blank	14366.33	997.6388	11015.40	17717.27	<.0001*
Day 14	Day 0	7563.33	997.6388	4212.40	10914.27	<.0001*
Day 7	Day 1	6679.67	997.6388	3328.73	10030.60	0.0002*

Comparisons with a control using Dunnett's Method

Control Group = Blank

Confidence Quantile

Idi	Alpha
2.90130	0.05

LSD Threshold Matrix

Level	LSD	p-Value
Day 3	71964	<.0001*
Day 7	40363	<.0001*
Day 1	33684	<.0001*
Day 14	19035	<.0001*
Day 0	11472	<.0001*
Blank	-2894	1.0000

Positive values show pairs of means that are significantly different.