Low-Cost Isothermal Amplification Microdevice for Rapid Colorimetric Detection Applied to Body Fluid Identification and Y-screening

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An-Chi T. Scott, Tiffany R. Layne and James P. Landers

PI
James P. Landers
Commonwealth Professor of Chemistry
Professor of Mechanical Engineering
Associate Professor of Pathology
University of Virginia

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SUMMARY OF THE PROJECT

1 MAJOR GOALS AND OBJECTIVES

While the mention of DNA amplification is automatically associated with thermocycling and the polymerase chain reaction (PCR), isothermal amplification methods are more applicable to qualitative assays owing to its specificity and the fact that the result can be read out colorimetrically with select dyes. Here, we describe why loop-mediated isothermal amplification (i.e., LAMP) should be considered for a variety of important forensic applications and, in particular, body fluid identification (bfID) and Y-screening. With bfID, accurate presumptive and confirmatory tests are essential for gaining contextual information for crime scene investigators yet suffer from poor reliability. With chemical-based tests, 'false positives' are not uncommon, there is concern over poor specificity, and many methods are either destructive to the sample and/or inhibitory to downstream processes. This has prompted a paradigm shift in which nucleic acids are utilized for screening body fluids. Even in the case where semen is identified and a sample subsequently extracted, there is substantial risk that the sample is non-probative due to an absence of male DNA. Commonly, samples are batched for screening using real time PCR due to the high cost of reagents/labor. Here, we explored a transcriptomic method based on isothermal amplification to rapidly identify body fluid including venous blood, menstrual blood, semen, saliva and, vaginal fluid – the common body fluids associated with the analysis. In addition, we sought to apply the same approach to male-specific DNA as a potential Y-screen.

2A RESEARCH QUESTIONS

The use of LAMP allows for colorimetric detection using an array of dyes and, hence, enables the use of inexpensive detection in the form of a smart phone or comparable camera. We show compelling evidence that the proposed method is as good or better than current presumptive and confirmatory testing. The setup not only eliminates human subjectivity, but also requires a small footprint and will be adapted to a 96-well format, thus presenting the possibility for portability and field-use. Once optimized, this method should be easily integratable into current forensic casework workflow and upon commercialization, should offer an inexpensive, yet reliable, alternative to existing bfID tests.

2B BACKGROUND

There is no question that PCR has dominated the DNA amplification landscape as a result of the single amplicon generated from thermocycling, and the ability for rapid generation of billions of copies of that amplicon. Isothermal amplification has lurked in the background for two decades[2] and, despite the allure of dodging thermocycling and heat denaturation, it has not been widely adopted. This is due, in part, to the need for more primers (4 or 6), and the fact that an amplicon of single size is not generated. Instead amplicons of a wide size range are generated covering a broad range of molecular size. However, isothermal amplification (e.g., LAMP) is ideal for

qualitative assays because it is highly specific (due to the large number of primer pairs), generates much more amplicon DNA than PCR, and successful amplification can be read out colorimetrically. Given these attributes, it is a clear that isothermal amplification be considered for a variety of important forensic applications – included in these is body fluid identification (bfID) and Y-screening.

With bfID, accurate presumptive and confirmatory tests are essential for gaining contextual information for crime scene investigators yet reliable assays are scarcely available. False positives results are not uncommon with (bio) chemical-based tests that lack specificity. In addition, many methods are known to be destructive to the sample and/or inhibit downstream processes. This has prompted a paradigm shift in which nucleic acids are utilized for screening body fluids. Even in the case where semen is identified and a sample subsequently extracted, there is substantial risk that the sample is non-probative due to an absence of male DNA. Commonly, samples are batched for screening using real time PCR due to the high cost of reagents/labor. Here, we propose an isothermal amplification method to rapidly identify body fluid including venous blood, menstrual blood, semen, saliva, vaginal fluid as well as male-specific DNA using colorimetric response and smart phone detection. The method will be amenable to implementation into current forensic casework protocols and offers an inexpensive yet reliable alternative to screening forensic samples. As will be seen preliminary data, we show strong evidence that the proposed method is superior to current presumptive and confirmatory testing in three fundamental ways. First, targeting messenger RNA (mRNA) for bfID has a high level of specificity derived from the use of multiple primer sets in the LAMP assay, and is inherently tissue-specific and human-specific. Evidence showing successful identification of blood, semen, and saliva from extracted mRNA sets the stage for the work outlined in this proposal to expand the range of body fluids that can be detected. Second, the simplicity of this screening method will minimally disrupt forensic labs performing DNA analysis. With abundantly-expressed mRNA co-extracted with DNA, only a small fraction of the sample (1 µL) is needed to perform reverse transcription-LAMP, preserving ample volume for additional analysis. Finally, the nature of the isothermal amplification massively reduces the complexity of instrument that we will design and build. With colorimetric detection an inherent part of the LAMP step, samples positive or negative for any 6 of the body fluids is readily identified by the smart phone 'app'. The setup not only eliminates human subjectivity, but also requires a small footprint and will be adapted to a 96-well format, thus presenting the possibility for portability and field-use.

There are multiple approaches to monitoring the progression of LAMP-based amplification. The simplest is visually sighting of the solution turbidity as magnesium pyrophosphate produced during amplification readily precipitates; drawbacks here are the subjectivity. Real-time turbidimeters are commercially-available, such as the LA-500 (Eiken Chemical Co.), but are being phased out due to other facile detection methods. Fluorescent indicators can be included in a LAMP reaction, and these, as expected, provide higher sensitivity. However, the need for fluorophores and more complex hardware increases the cost significantly. An alternative approach is to use colorimetric indicators that are compatible with LAMP[3, 4]. *New England Biolabs (NEB)* has perfected the use of phenol red (pH indicator) for LAMP, and the use of hydroxynaphthol blue (HNB, a metal indicator) is widely adopted. There is nothing that suggests this shouldn't be applicable to body fluid targets. The striking color change resulting from amplification enables easy visual detection of amplified reactions, however, this is subjective and

has modest limits of detection (LOD). Hence, we have created simple-to-use apps on smart phones[5] that allow for an objective, simple, accurate and sensitive color detection exploiting various parts of the color space (RGB, HSB). To allow for objective detection, we have integrated a simple, lost-cost (US$29.95) *Raspberry Pi* camera to monitor the color change throughout the course of the amplification process; this is analogous to a real-time PCR. The attribute of color adapted for this colorimetric analysis is hue (from the HSB color space), where each color is associated with a value between 0-255 in an 8-bit image. Hue values can, therefore, quantitatively reflect whether amplification due to the presence of a specific target has occurred, and to what extent. For example, with successful amplification, phenol red changes from pink (hue ~5) to yellow (hue ~30) as a result of acidification of the reaction mix. Recently, Krauss *et al* demonstrated the power of hue analysis by simultaneously detecting five colorimetric indicators associated with chemical reactions specific to total protein, albumin, cocaine, TNT, and Fe on a microfluidic platform[6]. In this work, all five analytes were detected by capturing images of the initial and final reaction mixtures, with hue analysis performed via *ImageJ* software. An advanced version of *ImageJ*, *Fiji*, combines scripting language to enable automated image analysis via algorithms[7], further enhancing potential high-throughput, real-time analysis.

In the typical forensic analysis workflow, after an evidentiary sample is submitted to a forensic laboratory, it will first be tested for body fluid identification (bfID). This is followed by DNA isolation and purification, quantitative polymerase chain reaction (qPCR), short tandem repeat (STR) amplification, and finally capillary electrophoresis to obtain an evidentiary DNA profile. Each sample is tested through presumptive bfID assays before more specific and/or sensitive confirmatory assays are carried out, though both types have major flaws. For instance, presumptive and confirmatory tests have not been developed for all body fluids and those that are available can be ridden with false positives and negatives due to cross-reactivity of enzymatic reactions. As for confirmatory tests, only blood and semen have well-developed kits for bfID. Furthermore, both types of tests can be laborious and subjective even for a trained analyst. For these reasons, there is an urgent need for a reliable alternative to identifying specific body fluids. We believe that a colorimetric (as opposed to fluorescence) approach to body fluid ID simplifies the assay and, in combination with exponential amplification cDNA emanating from RNA targets via isothermal amplification, holds tremendous potential for a cost-effective assay. Moreover, the speed, sensitivity, and specificity of a LAMP-based method provides is a significant improvement over current bfID methods which could revolutionize forensic analysis of sexual assault investigations with confirmed reliable fluid identification.

3 RESEARCH DESIGN

Presumptive and confirmatory body fluid identification (bfID) tests are the first analyses performed in a long series of evidentiary workflow. The results ultimately determine the fate of a piece of evidence, by providing clues for investigators as to whether it is deemed useful or simply background noise. Currently, bfID is primarily carried out by enzymatic- or immunological-based assays. Blood, saliva, and semen are the most widely-available and routinely executed tests today. However, the current tests possess some combination of characteristics that include antiquated, slow, generate false positives, lack of specificity, destructive to the sample and cause

inhibition of downstream processes. Furthermore, these tests can be laborious and subjective even for a trained analyst. For these reasons, there is an urgent need for a reliable alternative method to identifying body fluids, and to expand the types of body fluids that are common in crime scenes. To address these shortcomings, we proposed the development of a non-fluorescent mRNA-based bfID method that would exploit a novel amplification method called Loop-mediated AMPlification (LAMP). LAMP allows for highly specific amplification of the target nucleic acids with higher efficiency than a typical PCR reaction and, most importantly, under isothermal conditions.

The approach focused on a body fluid ID panel that included venous blood, saliva, semen, vaginal fluid, and menstrual blood from forensically-sized samples (~2µL or less). Additionally, using the same LAMP approach, a rapid Y-screen method was optimized using a crude lysis coupled with Y-amelogenin-specific LAMP primers. An advantageous feature of LAMP is that amplification can be directly linked with dye color change for colorimetric monitoring of the reaction, which can either be visualized (naked eye) or quantified using a simple camera setup. In addition to attempting to define a LAMP-based *five-body fluid+Y-screen assay*, we engineered – the *iLAMP* instrument – an integrated system capable of controlling temperature hold during amplification, as well as executing image analysis in real-time to quantify color changes as amplification progresses. Although *iLAMP* was initially viewed as an 'endpoint detection' or 'qualitative' system (color change = presence of body fluid), it is clear from the colorimetric profiles that are peppered throughout this report, that the ability for real-time monitoring of color change is possible. And while the equivalent of a qPCR fluorescence plot may be ambitious, the potential capability for 'semi-quantitative' measurement is, in fact, achievable. In this two-year project, we had success detecting saliva (Sa) and vaginal fluid (VF), alone or in mixtures, and excellent performance with the Y-screen. Detecting venous blood (VB) and semen (Se) was only modestly successful, and will require further optimization in order to have a robust a 4-body fluid panel.

3A METHODS

Sample Collection

All donated de-identified body fluid samples containing venous blood, saliva, semen, vaginal fluid or menstrual blood were collected in accordance with the University of Virginia's International Review Board (IRB) policies. The vaginal fluid and menstrual blood samples were collected on sterile cotton swabs, dried over-night, then stored in a -20 °C freezer until analysis. The saliva and semen samples were collected in sterile specimen containers, aliquoted into 50 and 20 µl increments, respectively, and stored in a -20 °C freezer until analysis. Saliva samples were also collected via a sterile cotton swab and 30 sec of swabbing the inside cheek, drying for 24 hours and stored at room temperature until analysis. The venous blood was received de-identified from the University of Virginia Medical Hospital collected via a standard venipuncture technique as a part of routine care and treated with 5.4 mg of K_2EDTA for anti-coagulation and stored in a 4 °C refrigerator. Once received, the blood samples were aliquoted into 50 µl increments and stored in a -20 °C freezer until analysis.

Y-Screen Sample Preparation and Lysis

On the day of analysis, semen aliquot was thawed, thoroughly mixed, and serially diluted in DNase-free water to dilution factors (DF) of 50, 500, and 5000. The lysis was carried out using the *forensic*GEM *Sexcrime* kit (ZyGEM, NZ) in 100 µL following the manufacturer's protocol (1x Orange Plus Buffer, 2 µL *forensic*GEM, 10 µL ACROSOLV), as well as a modified protocol containing 0.5x Orange Plus Buffer ('modified lysis'). Lysis was also prepared without *forensic*GEM and ACROSOLV as a protease-free control, to demonstrate the integrity of the sperm cells. Each lysis condition was prepared in triplicate. All samples were incubated at 52°C for 5 min, 75°C for 3 min, and 95°C for 1 min. The lysates were stored at -20°C until analysis.

Mock swabs were prepared after estimating the cell numbers by hemocytometer post SYTO-11 (Thermofisher, USA) staining. Semen was serially diluted to 40, 400, or 4000 cells in 100 µL when deposited onto each cotton swab (Puritan, USA). Female buccal cells were diluted to 400 cells in 100 µL when deposited onto a cotton swab. All dilutions were prepared in duplicate and the swabs dried at room temperature overnight. The dried swabs were each assigned a de-identified number, sealed in a 1.5 ml microcentrifuge tube and stored at -20°C until analysis 2 weeks later. Sample lysis was performed on a quarter swab cut from each numbered swab using the modified *forensic*GEM *Sexcrime* protocol mentioned above. The lysate was separated from the swab cutting by piercing the bottom of the PCR tube and spinning the liquid into a new PCR tube. The samples were subjected to DNA quantification and LAMP analysis.

For specificity studies, human female DNA was obtained from de-identified buccal donations collected using a FLOQSwab (COPAN, Italy). The whole swab was lysed in *forensic*GEM Saliva kit (ZyGEM, NZ) in 100 µL containing 1x Blue Buffer, 2 µL *forensic*GEM, and incubated at 75°C for 15 min and 95°C for 5 min. Blood collection from mouse, pig, rabbit, and rat was approved by the UVA ACUC for diagnostic and research purposes under the animal protocol used in this study. DNA was prepared using a QIAamp DNA mini kit (Qiagen, Germany) and stored at -20°C until analysis as described in Duvall et al.[8]

For the dilution studies, semen aliquots (Donor A) were pooled to have enough for the study. After thorough mixing, semen was diluted to 1:2 (1-part semen, 1-part water) and 1:10 (1-part semen, 9-parts water). The 1:10 dilution was then further serially diluted to 1:100 and 1:1000. One hundred microliter of neat or diluted semen was deposited onto duplicated swab. Two female buccal samples were collected for negative control. The swabs were dried overnight at room temperature (RT). The swab handles were threaded through a 1.5 mL Eppendorf tube with bottom cut off and stored at -20°C until analysis. The second dilution study was prepared using semen from Donor B and C, which gave an estimated cell count of 91,000 and 41,000 cells/µL, respectively. Semen was first diluted to 1:100, and subsequently diluted to 1:1000, 1:2000, 1:5000, and 1:10000. One hundred microliter of each dilution was deposited onto duplicated swabs, and dried and stored as described above.

Mock sample set one was prepared 'blind' without the operator knowing the sample content. Retrospectively, the content was revealed to range from male urine (neat), male buccal cells (neat), female buccal cells (neat), and semen (1:20 and 1:160). Mock sample set two contained mixed female buccal and male sperm cells (semen). The cell concentration was estimated by staining with SYTO-11 and counted on a hemocytometer. Cells were then diluted to have the desired total cells in 100 µL to be deposited onto each swab. For example, "M10+F100" sample was calculated to have 10 sperm cells and 100 female epithelial cells for each quarter swab

cutting. Post-coital (PC) swabs were donated with self-reported post-coital interval (PCI). Mock sample set three contained the most realistic mimics, using vaginal swabs in B001, B002, and B009 samples instead of buccal cells. Dilute semen was then deposited onto the swabs or jeans. This sample set also contained four PC swabs from different donor couples, and six PC swabs with PCI from 24h to 144h from one donor couple. Mock sample set one and two were stored at -20°C for up to one year. Mock sample set three were stored at RT from one week to four years.

One quarter of the swab was cut on a clean surface using an Xacto knife and placed into a PCR tube. Three cuttings were prepared from each swab for triplicate analysis. A reaction mixture of lysis reagent was prepared with the modified lysis method. Each 100-µL reaction mix contained 0.5X Orange Plus Buffer, 2 µL forensicGEM, and 10 µL Acrosolv. The sample was vortexed prior to incubation at 52°C for 5 min, 75°C for 3 min, and 95°C for 1 min. Once lysis is complete, the tube was cleaned on the outside and pierced with a syringe needle at the bottom while inverted, and placed into a 0.6 mL Eppendorf tube. The needle was reused in the dilution study, working from most dilute sample first. The needle was replaced for each mock sample. The tube ensemble was spun at 10,000 RPM for 60 sec to separate the lysate from the swab. The lysate was stored at -20°C.

RNA Isolation

The samples were lysed using a previously published protocol[9]. In a centrifuge tube, 350 µl of RLT buffer (Qiagen) was combined with 90 µl RNA-free water (Growcells, USA), 10 µl Proteinase K (Qiagen), and 4.5 µl of B-mercaptoethanol (Sigma Aldrich, USA). For each of the fluids, 50 µl of venous blood, 2 µl of seminal fluid, whole swab of vaginal fluid or menstrual blood, or 100 µl of saliva were added to the centrifuge tube. Each sample was incubated at 56 ^{o}C for 10 minutes. Each swab sample was then placed in a 0.5 mL tube that was punctured with a 21-gauge needle in the bottom of the tube. The tubes were placed in 1.5 mL microcentrifuge tubes and centrifuged for 1-2 seconds at maximum speed. Remaining fluid from the swab samples spun through to the 1.5 mL tube and were combined with the original lysed sample. All of the lysed samples were extracted using Qiagen's RNeasy Mini kit. The manufacturer's protocol was followed after lysing of the samples. There was an on-column DNase Digestion with RNase-Free DNase performed per manufacturer's protocol (Qiagen). The samples were extracted in 50 µl of RNase-free water and kept in a -80 °C freezer.

DNA Quantification

ZyGEM-derived DNA was quantified via Taqman qPCR targeting the human TPOX marker, sequences previously published[10]. Each 15 µL reaction consisted of 1x PerfeCTa supermix low ROX (Quanta, USA), 0.3 µM forward and reverse primers (Eurofins, USA), 0.2 µM probe, and 2 µL of unknown DNA. Primer sequences for forward: CGGGAAGGGAACAGGAGTAAG; reverse: CCAATCCCAGGTCTTCTGAACA; and probe: FAM- CCAGCGCACAGCCCGACTTG-TAMRA. Purified human DNA G1471 (Promega, USA) was used as standards ranging from 0.016 – 10 ng/µL. Samples were run in duplicate on ABI 7500 fast Real-Time PCR System (Thermofisher) at 95°C for 3 min, then 40 cycles of 95°C for 10 sec and 60°C for 45 sec. Quantification cycle (Cq) was automatically determined by the 7500 software v2.3 (Thermofisher). In the single blind study, DNA quantification was performed using Plexor HY System (Promega, USA) to estimate autosomal and male DNA simultaneously. Each 20 µL Plexor reaction consisted of 1x MasterMix,

1x Primer/IPC Mix, and 2 µL template. Plexor HY Male Genomic DNA Standard was prepared according to protocol, ranging from 0.0032 ng/µL to 50 ng/µL. Samples were run in duplicate on ABI 7500 fast Real-Time PCR System at 95°C for 2 min, then 38 cycles of 95°C for 5 sec and 60°C for 35 sec, and finally a melt curve analysis. Data analysis was performed with Plexor Analysis Software v1.6.0 (Promega, USA). Purified DNA from animal origin was quantified using a NanoDrop 1000 spectrometer (Thermofisher).

Primer information

All of the LAMP primer sets were designed using PrimerExplorer V5 (http://primerexplorer.jp) and purchased from Eurofins Genomics LLC. The blood primer set was designed from the human β-hemoglobin messenger RNA sequence (HBB; NM_000518.5). The semen primer set was designed from the human semenogelin 1 messenger RNA sequence (SEMG1; NM_003007.4). The saliva primer set was designed from the human histatin 3 messenger RNA sequence (HTN3; NM_000200.2). The vaginal fluid primer sets were designed from human beta-defensin 1 (DEFB1; NM_005218.4) and cytochrome P450 family 2 subfamily A member 7 pseudogene 1 (CYP2B7P1; NR_001278.1). The menstrual blood primer sets were designed from human left-right determination factor 2 (LEFTY2; NG_008118.1), human matrix metallopeptidase 10 (MMP10; NM_002425.2) and human matrix metallopeptidase 11 (MMP11; NM_005940.4). For Y-screen, the target was human Y-Amelogenin sequence (NC_000024.10).

Colorimetric Loop-mediated Isothermal Amplification

The New England Biolabs (NEB; USA) Colorimetric LAMP kit was used for experiments according to the manufacturer's instructions. Total reaction volumes were reduced to half reaction (12.5 µL) and consisted of 6.25 µl 2X WarmStart Colorimetric Master Mix (final 1X concentrations: low-Tris reaction buffer with 8 U Bst 2.0 WarmStart DNA Polymerase, WarmStart RTx, 8 mM MgSO4, 1.4 mM dNTP each, Phenol Red), 1.25 µl of various concentrations of primers, and 3.75 µl of DNase-RNase-free water. Approximately 1.25 µl sample volumes were added to reaction volumes. The recommended primer concentrations (1X) given by the manufacturer are 0.2 µM for F3 and B3, 0.4 µM for LF and LB, and 1.6 µM for FIP and BIP, but the primers were also tested at 0.5X (F3/B3: 0.1, LF/LB: 0.2, FIP/BIP: 0.8 µM each), 0.75X (F3/B3: 0.15, LF/LB: 0.3, FIP/BIP: 1.2 µM each) and 1.5X (F3/B3: 0.3, LF/LB: 0.6, FIP/BIP: 2.4 µM each). The samples were amplified at 63, 65 or 67 °C using a Veriti Thermal Cycler (Thermo Fisher) or in-house built heating chamber. When needed, LAMP reactions were examined on an Agilent 2100 Bioanalyzer using DNA 1000 series II kits (Agilent Technologies, Santa Clara, CA) for confirmation of amplification. For sensitivity studies, the sample RNA lysates were quantified before LAMP using RiboGreen™ (ThermoFisher) according to manufacturer's protocol on a Nanodrop 3300 (ThermoFisher) and quantified by average based on triplicate analysis. The samples were diluted to various concentrations of Total RNA for LAMP testing.

Non-colorimetric LAMP reagents were also purchased from NEB unless otherwise specified. Each typical 12.5 µL reaction contained 1x Isothermal Amplification Buffer, 8 mM $MgSO_4$, 1.4 mM each dNTP, 8U *Bst* 2.0 WarmStart, and 2 µL template. Colorimetric indicators were prepared according to Scott *et al.*[11] The final concentration of each indicator was 120 µM for hydroxynaphthol blue (HNB), 0.1 mM leuco crystal violet (LCV), 0.004% (w/v) malachite green (MG), or 25 µM calcein. Fluorescent LAMP reactions were run on the ABI 7500 Fast with 1 µM SYTO-9 (Thermo Fisher) using the FAM filter set. LAMP primer sequences are shown below in

Table 1. Each LAMP assay was performed with duplicated no template control (NTC) and positive control (Pos) using purified human male DNA G1471 (Promega, USA) at 10 ng/µL. Sensitivity study was done by serially diluting G1471 in water to achieve a range of 25 pg/µL to 1 ng/µL. LAMP was performed at 63 or 65°C with visual checks between 30 and 60 min at 10-15 min intervals. A positive LAMP reaction is indicated by the transition in HNB color from violet to blue or a transition in phenol red color from pink to yellow. Positive and specific LAMP reaction was confirmed by running the product on the DNA chip in the Bioanalyzer 2100 (Agilent Technologies, USA) wherever suitable.

P30 Testing

ABAcard p30 assay (Abacus Diagnostics) was used for the identification of semen with manufacturer's protocol. One quarter of the swab was removed with an Xacto knife and placed into a 1.5 mL Eppendorf tube. Seven hundred and fifty microliters of Extraction Buffer was added to the sample and incubated at 4°C for 2 hours. The samples were centrifuged at 5,000 RPM for 3 min, and 300 µL of supernatant transferred to a new tube. Two hundred microliters were then transferred onto the assay strip and allowed 10 min to develop. A line at the control ("C") indicated a valid test, whereas a line at the test ("T") indicated positive for semen. Assay strips were scanned using an Epson Perfection V100 desktop scanner for record keeping.

STR Analysis

PowerPlex Fusion System (Promega Corp., USA) was used for autosomal STR analysis. A reaction mix was prepared containing 1x Fusion mater mix (MM) and 1x Fusion primer mix (PM) in a 12.5-µL reaction. One microliter of DNA sample was added to the reaction without normalization. Each amplification was prepared with 1 ng 2800M DNA as positive control (Pos) and water as NTC. The reaction was incubated at 96°C for 1 min, then 28 cycles of 94°C for 10 sec, 59°C for 1 min, and 72°C for 30 sec, and a final extension at 60°C for 10 min. One microliter of post amplification product was heat-snap-cooled in the presence of 9.5 µL Hi-Di formamide (HDF) and 0.5 µL WEN internal lane standard (ILS) by heating to 95°C for 3 min, and immediately chilled in ice water. The mixture was electrophoresed on an Applied Biosystem PRISM 3100 xl Genetic Analyzer (Thermofisher). The fragment analysis data files (*.fsa) were exported to GeneMarker v2.8.2 (SoftGenetics, USA) for allele calling and additional analysis.

iLAMP Integrated System for Real-time Colorimetric Detection

The instrument hardware component and operating software are detailed in the Section 4C as part of the development. Briefly, the integrated LAMP instrument, iLAMP, has a 3D-printed enclosure with four major compartments: main compartment to house the sample plate, lid (top chamber) for lighting, back chamber for heating, and bottom chamber for electronics and the Raspberry Pi (Rpi) camera v2. The instrument is controlled via a laptop using a graphic user interface (GUI) in LabVIEW (National Instruments, USA). Image analysis is performed in a custom script in FIJI (NIJ). During heater optimization, temperature was recorded using Type T thermocouples (Physitemp, USA) connected to a VersaLog 8 channel thermistor data logger (MicroDAQ, USA). Using Fiji[7], the image is analyzed for the hue value. For phenol red LAMP reactions, the image is tinted (hue scale rotated) so all red values congregate at the upper end of the scale with the yellow values at the bottom. First, the image is tinted by changing the blue channel to 0-190 and yellow channel to 40-255. Second, the image is analyzed for hue in a circle

with radius of 15 pixels and exported to a .csv file. The .csv file contains sample number, area of circle, mean of hue, minimum hue detected, and maximum hue value detected.

Table 1 - *Amplification primers. A hyphen in LAMP primers indicate the connector in between F2 and F1c, which has a tetra thymine insert if a linker is included.*

	Target		Sequence
Taqman PCR	TPOX	F	CGGGAAGGGAACAGGAGTAAG
		R	CCAATCCCAGGTCTTCTGAACA
		Probe	FAM-CCAGCGCACAGCCCGACTTG-TAMRA
LAMP Nogami et al.[1]	Y-Amelogenin	F3	GGTCCCAATTTTACAGTTCC
		B3	CTGGTCAGTCAGAGTTGAC
		FIP	AATCCGAATGGTCAGGCAGG-CCAGTTTAAGCTCTGATGGTT
		BIP	GACTCTTTCCTCCTAAATATGGCTG-TTTTGCCCTTTCATGGAAC
		LF	GGTGCTGGAGCAACACAG
LAMP ID50	Y-Amelogenin	F3	ATTTTACAGTTCCTACCATCAG
		B3	GACTGACCAGCTTGGTTC
		FIP	CTTCCCAGTTTAAGCTCTGAT-TCCTGCCTGACCATTCGGAT
		BIP	TGACTCTTTCCTCCTAAATATGGCT-TTCCATGAAAGGGCAAAAAG
LAMP Loop44	Y-Amelogenin	LF	CTCAAGCCTGTGTTGCTCCA
		LB	CATGAACCACTGCTCAGGAAGG
LAMP Loop82	Y-Amelogenin	LF	CTCAAGCCTGTGTTGCTCCA
		LB	CATGAACCACTGCTCAGGAAGG
LAMP	Venous Blood	F3	CCTCAAGGGCACCTTTGC
		B3	TTGTGGGCCAGGGCATTA
		FIP	CGTTGCCCAGGAGCCTGAAGTTTTACTGAGTGAGCTGCACTGT
		BIP	GGTCTGTGTGCTGGCCCATCTTTTCCAGCCACCACTTTCTGAT
		LB	CTTTGGCAAAGAATTCACCC
		LF	AGGATCCACGTGCAGCTTGT
LAMP	Semen	F3	TCTCATGGGGGATTGGAT
		B3	CATCTCAGAAACATCACAGAA
		FIP	GTTTCGGTCGTTGTTAAGCTGTTGTTTTTTAATTATAGAGCAGGAAGATGACAG
		BIP	TAAACCTACCATTCGGTAACCATGTTTTCACTGAGGTCAACTGACA
		LB	GAAAGGATGGACCAATATCAAG
LAMP	Saliva	F3	TTGGCTCTCATGCTTTCC
		B3	GGTATGACAAATGAGAATACACG
		FIP	GATGTGAATGATGCTTTTCATGGAATTTTCTGGAGCTGATTCACATGC
		BIP	ATTGATATCTTCAGTAATCACGGGGTTTTAGTCCAAAGCGAATTTGC
		LB	CATGATTATGGAGGTTTGAC
		LF	TATACCCATGATGTCTCT
LAMP	Vaginal Fluid (CYP2B7P)	F3	GCTTGATGACCGAGCCAA
		B3	GTCAGGATTGAAGGCGTCTG
		FIP	AATGTGGGGCACACCCATGGGCCATACACAGAGGCAGTC
		BIP	TTCTGAGGGTACACCATCCCCATCAAAGTAGTGTGGGTCACG
		LB	CGGAAGTATTTCTCATCCTGAGCA
		LF	GTCAGCAAATCTCTGAATCTCACGG
LAMP	Vaginal Fluid (HBD1)	F3	CCTGAAATCCTGGGTGTTGC
		B3	AAGATCGGGCAGGCAGAA
		FIP	CCACCTGAGGCCATCTCAGACATTTTCCAGTCGCCATGAGAACTTC
		BIP	ACTTTCTCACAGGCCTTGGCCTTTTGAGACATTGCCCTCCACTG
		LB	GATCTGATCATTACAATTGCG
		LF	AGCAGAGAGTAAACAGCAGAA

3B ANALYTICAL RESULTS

3.B.1 BODY FLUID ASSAY DEVELOPMENT

3.B.1.a LAMP and Hue Values from Image Analysis

Loop-mediated **AMP**lification (LAMP) is a method that exploits up to 3 pairs of primers to specifically amplify a nucleic acid target under isothermal conditions. It has the unique ability to amplify at higher temperatures (60-68°C), inherently providing enhanced specificity and amplification efficiency. The primer sets identify specific regions of a target, with the option for adding in an additional set of loop primers, which allows for more annealing sites and, thus, faster amplification. Briefly, upon annealing and elongation of inner primers, the outer primers anneal behind the inner primers, followed by strand displacement amplification via the polymerase, thus releasing the product from the inner primer. With the incorporation of the additional two primers, a dumbbell-like structure is formed and exponential amplification occurs. Due to the nature of LAMP, the resultant amplification products cover a range of fragment sizes, but all fragments, regardless of size, contain the desired target sequence. An additional advantage is that effective LAMP can be detected using colorimetric indicators for simplicity.

Grounding our approach to the detection of successful amplification is a path that avoids fluorescence and, instead, involves an indicator-mediated color change that accompanies successful isothermal amplification. With multiple (up to 3) primers sets required for each fluid target, failure of any of the primers to find the specific target sequence(s) leads to no (zero) DNA amplification. Moreover, if the target is present, successful amplification can not only be detected, it can be monitored in a semi-quantitative manner using color indicators such as hydroxynaphthol blue (HNB; metal indicator) or phenol red (PR; pH indicator). **Figure 1** provides a graphical description of this process. For HNB, as magnesium becomes increasingly coordinated with a pyrophosphate to form a less soluble complex, the decrease in magnesium concentration, leads to a color change of HNB from violet to blue. For PR, every nucleotide incorporated into the

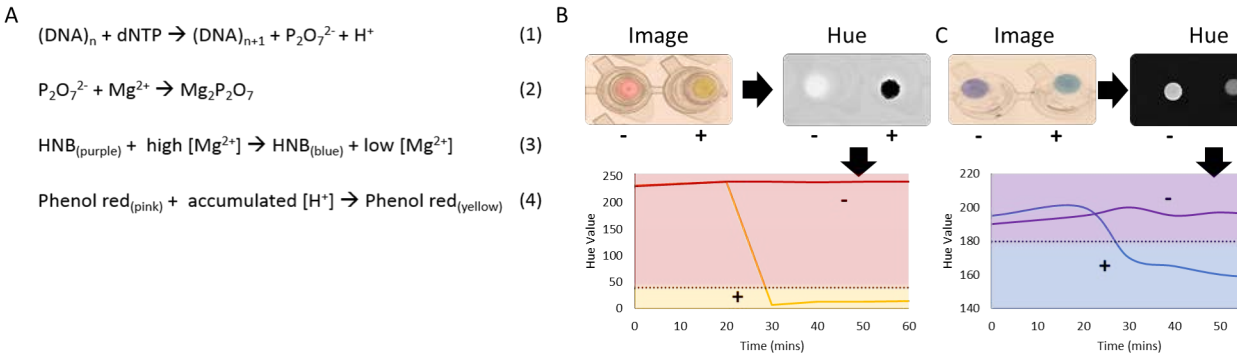

Figure 1 - *The reaction mechanism for color indicator detection of a successful LAMP amplification. (A) Rxn 1: Nucleotide incorporation polymerization results in the rapid accumulation of pyrophosphate and protons which provide the underlying mechanism for color change. Rxns 2-3: As the insoluble magnesium pyrophosphate increases during amplification, magnesium concentration lowers, thus HNB turns from a violet color to blue. Rxn 4: As the pH decreases during amplification, phenol red changes from pink to yellow. (B-C) Colorimetric analysis of phenol red and HNB color transition using hue, and the semi-quantitative plot of a positive and negative reaction.*

growing amplicon chain generates a hydrogen ion, decreasing pH, thus changing the color of PR from pink/red to yellow. Since both indicators involve a change in color, the 'shade' of the color

(hue value) can be monitored as an analysis method. For this research, image capture at various time points for hue analysis allows for defining presence/ absence) of any one of five body fluids.

Hue as the shade of a particular color has values that range from 0 to 255; thus, the red and yellow colors would have very different values (**Figure 2A**). The red values were in the range of 0-15 or 240-255 and the yellow values were 25-40. When averaging the red color values, the standard deviation can be very large depending on where the hue values fell for various reactions. Due to the split in the range for the red color, the hue color scale needed to be rotated to allow for a single range for the red color. While there are various ways to do this, this method used tinting of the image. This change rotated the hue color scale to allow for all red values to be between 230-255 and yellow values between 5-40. As shown in **Figure 2B**, the tinted version has a pinker color than the original image. When these images are transformed into hue grayscale, the red color becomes white and the yellow color becomes black. To define a threshold for a positive reaction, >100 reactions were averaged to define average positive and negative values, and a range calculated as three standard deviations above and below (**Figure 2C**). The hue values were taken from LAMP reactions across all 96 wells, which shows how the hue value minimally changes. Using this threshold, the LAMP reactions were determined to be a positive or negative result.

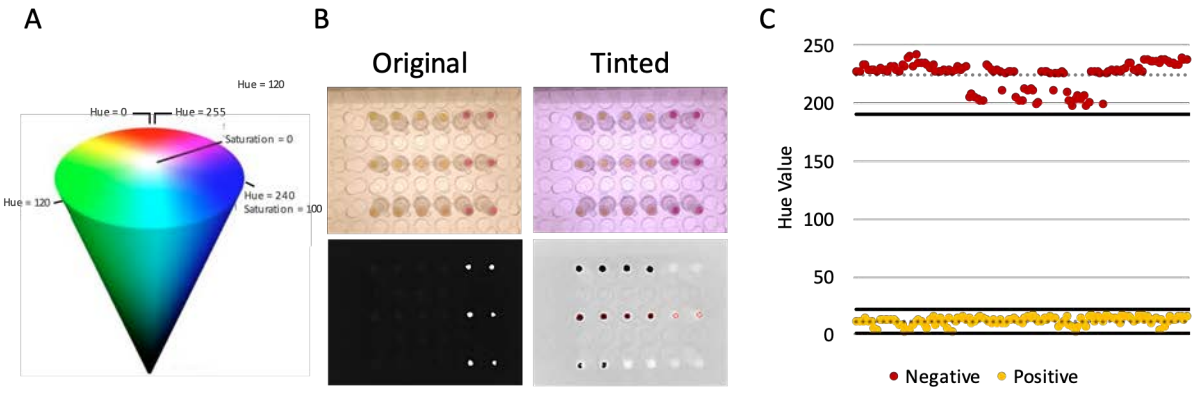

Figure 2 *- Image Analysis of LAMP amplification of body fluids with positive and negative ranges. (A) After capture, the images are tinted and then transformed into hue. An in-house macro crops the middle portion of the tubes and analyzes the reactions. (B) Dotted lines mean averages for positive or negative hues (n=125). respectively. The black lines are 3 standard deviations above or below the averages and show the ranges for both positive and negative hue values.*

3.B.1.b Blood, Semen, Saliva LAMP

The first step in defining a colorimetric assay for body fluids was to optimize the amplification conditions of mRNA targets in blood (VB), saliva (Sa), and semen (Se) by using loop-mediated isothermal amplification (LAMP). While LAMP uses up to six different primers (3 sets) to amplify a specific genomic target, each primer set was tested for target mRNA amplification specificity, and then tested with other body fluids to reveal cross reactivity as shown in **Figure 3**. One primer set for each of the fluids was used based on previous research efforts, and LAMP was optimized to achieve the shortest amplification time without compromising efficiency. Among the

parameters tested were temperature, primer concentration and total volume of the LAMP reaction.

Since this LAMP kit has never been utilized for forensic purposes, we tested a number of parameters to show effective amplification of the targets. **Figure 4** shows studies using all three primer sets with varied amplification temperature to determine whether a universal amplification temperature could be identified; this is critical for target specificity and for minimizing non-specific amplification (NSA). Since LAMP utilizes more primers than PCR, there is inherently, a higher probability of non-specific annealing via primer-dimers. Hence, known targets for each fluid were used as positive controls, and this resulted in adequate amplification for color change within 40 minutes (dark bars) in all three primer sets at 63 and 65 °C. The negative controls were the LAMP reagents with only PCR-grade water.

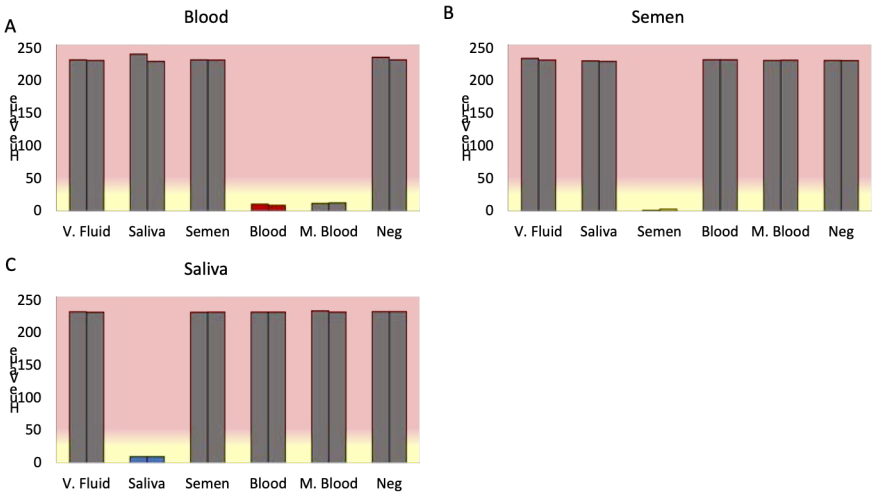

Figure 3 - *Specificity testing of body fluid primer sets in LAMP via colorimetric analysis. Each bar represents a replicate.*

Of the three body fluids, the VB primer set was the most sensitive to contamination and/or non-specific amplification. This is shown in **Figure 4A** where the negative controls were observed to changed color at 63 and 65 °C, with one of the two replicates changing color at 67 °C. Following analysis of the amplified products by microchip electrophoresis (Bioanalyzer), the color change was clearly due to NSA, theoretically from primer-dimer formation. While this is a problem, varying other components in the LAMP reaction may reduce the occurrence of non-specific amplification. For both the Se and Sa primer

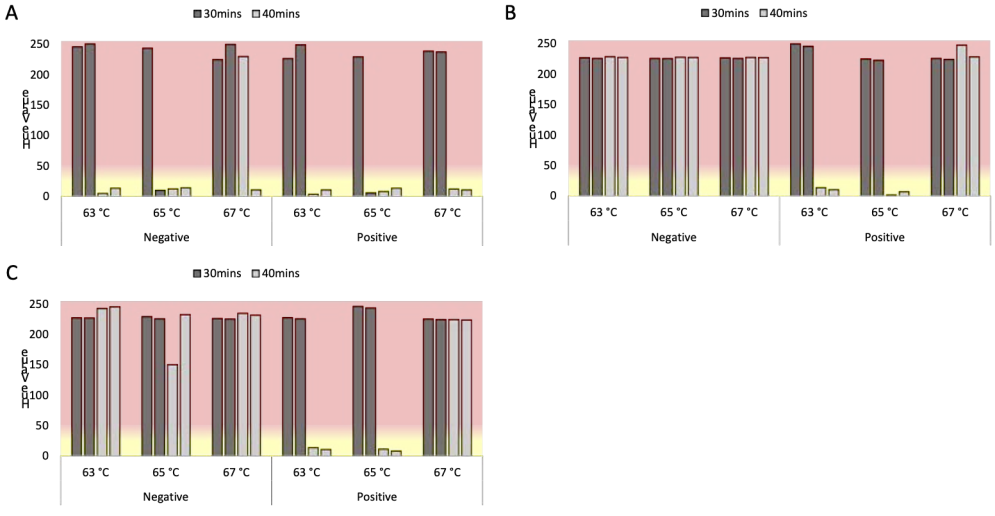

Figure 4 - *Temperature optimization for LAMP assay. For VB (A), Se (B), Sa (C), a positive and negative was amplified at three temperatures in duplicate to ensure no non-specific annealing was present.*

sets, the negative controls did not change color at any of the temperatures tested after 30 minutes of heating (**Figure 4B-C**). However, the positive controls changed color at 63 and 65 °C after 40 minutes, but did not change color at 67 °C. From these results, we decided to continue testing LAMP at 63 °C.

One of the goals of this research was to have a 'time-to-positive result' (T_{PR}) of ~30 minutes for all targets. To achieve this, the concentration of the primers in each set were varied for each of the LAMP reactions. The recommended 1X concentrations given by the manufacturer are 0.2 µM for F3 and B3, 0.4 µM for LF and LB, and 1.6 µM for FIP and BIP. Since the VB primer set had previously been shown to be susceptible to NSA in the negative controls, the primer concentrations were decreased to minimize NSA-induced color change during LAMP until beyond 30 minutes (**Figure 5**). While this may adversely affect the VB LAMP color change T_{PR}, the positive control reaction time was ultrafast, so that the decrease in primer concentration had less of an effect on T_{PR} for VB. The primer concentrations tested were at 0.75X (F3/B3: 0.15, LF/LB: 0.3, FIP/BIP: 1.2 µM each) and 0.5X (F3/B3: 0.1, LF/LB: 0.2, FIP/BIP: 0.8 µM each). In contrast, with the Se and Sa primer sets, the positive control was not amplifying inside 30 minutes, and the negative controls showed no signs of NSA. Given this, the primer concentrations were increased to 1.5X (F3/B3: 0.3, LF/LB: 0.6, FIP/BIP: 2.4 µM each) to achieve the necessary T_{PR} goal. Previously, these primers were used with an *Eiken Loopamp LAMP kit* (Shanghai, China) at a slightly higher concentration with no NSA detected[9]. From the literature[12-14] and our previous testing[9], NSA did not appear to be a problem with higher primer concentrations, and all primer concentrations were tested at 63 °C in order to attain a T_{PR} of 30 minutes.

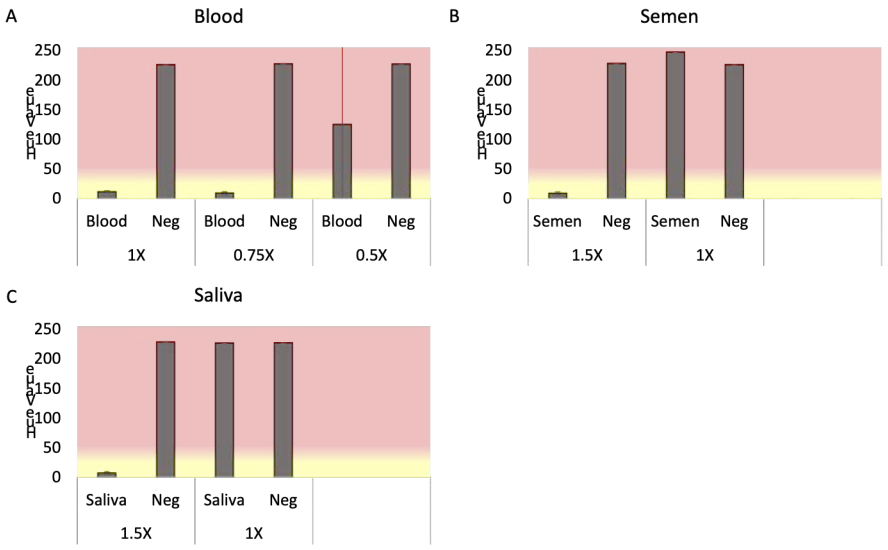

Figure 5 - *Primer concentration optimization for LAMP assay. For VB (A), Se (B), Sa (C), each fluid bar is an average of two replicates from two different donor samples and each negative bar is an average of two replicates.*

In evaluating the primer concentration results for the VB primer set, 1X and 0.75X allowed for amplification of all positive controls by 30 minutes, but the 0.5X concentration led to variable results. In addition, all of the negative controls for the VB target were not amplified by 30 minutes. Based on these results, a 0.75X primer concentration for the VB primers was carried forward with the remaining experiments. Using this primer concentration allowed for ample amplification of the VB target in samples, while keeping NSA from interfering with effective sample analysis.

3.B.1.c Vaginal Fluid and Menstrual Blood LAMP

Vaginal fluid (VF) and menstrual blood (MB) were also tested using LAMP. These fluids are more difficult to identify due to: 1) a scarcity of known targets that are specific to those tissues, and 2) similarity in composition to other fluids, e.g., menstrual blood and venous blood. A number of approaches were pursued to define a primer sets specific for VF and MB cDNA, without amplifying nucleic acids present in other fluids. The literature provided a number of viable genetic targets for both fluids[15-20], and multiple primer sets identified for at least two unique genetic targets per fluid. All primer sets were tested at 63, 65, 67 °C with incorporated negative controls and against other fluids. However, off-target amplification (NSA) provided serious challenges here. Non-specific amplification can stem from primer self-annealing or annealing to other targets containing partial homologous sequences. As done in previous studies, NSA was confirmed through microchip electrophoresis (Bioanalyzer) analysis of the amplified LAMP products. Since LAMP amplicons build off an initial key-like structure, if the profiles from the Bioanalyzer do not display the same amplicon pattern as the positive control, the result was deemed to be NSA. Examples of this are shown in **Figure 6** where the LAMP banding patterns for VF or MB relative to the negative control or the other body fluid. Many of the primer sets tested showed either amplification with other fluids (i.e., MB primer set amplifying VB) or NSA in a negative control or with the other fluid (i.e., VF primer set amplifying a (-) control).

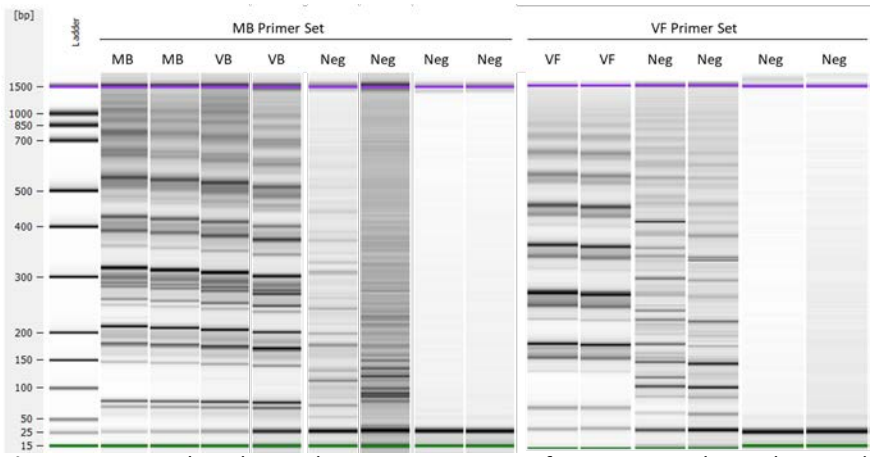

Figure 6 *- Microchip electrophoretic separations of various samples with VF and MB primer sets. The MB primer set shows fluid non-specificity by on-target amplification in VB and primer non-specificity by off-target amplification in some negative controls. The VF primer set shows primer non-specificity by off-target amplification in some negative controls.*

This led us to explore the use of chemical agents that help reduce the number of mismatched base pairs in an amplification method. Diethyl formamide (DEF) is one such agent, and it was evaluated for effectiveness at minimizing NSA with the VF and MB primer sets. Presumably, the non-specific targets we observe result from primer dimers or binding to partially complimentary sequence in the RNA. Based on the results in **Figure 7,** when 3% DEF was used (recommended concentration), there was an immediate color change from red to yellow. This was problematic given the importance of color change as a detection mode. Since the color change is pH-based, we titrated in Tris and increased the reaction temperature from 63 to 65 °C to combat the effects of DEF. As given in **Figure 7**, the use of DEF reduced NSA, and avoided a false color change from red. While this presented a viable option for moving forward, it was a Band-Aid and not a comprehensive solution to the NSA problem. Hence, we chose further our search for a primer set that minimized NSA.

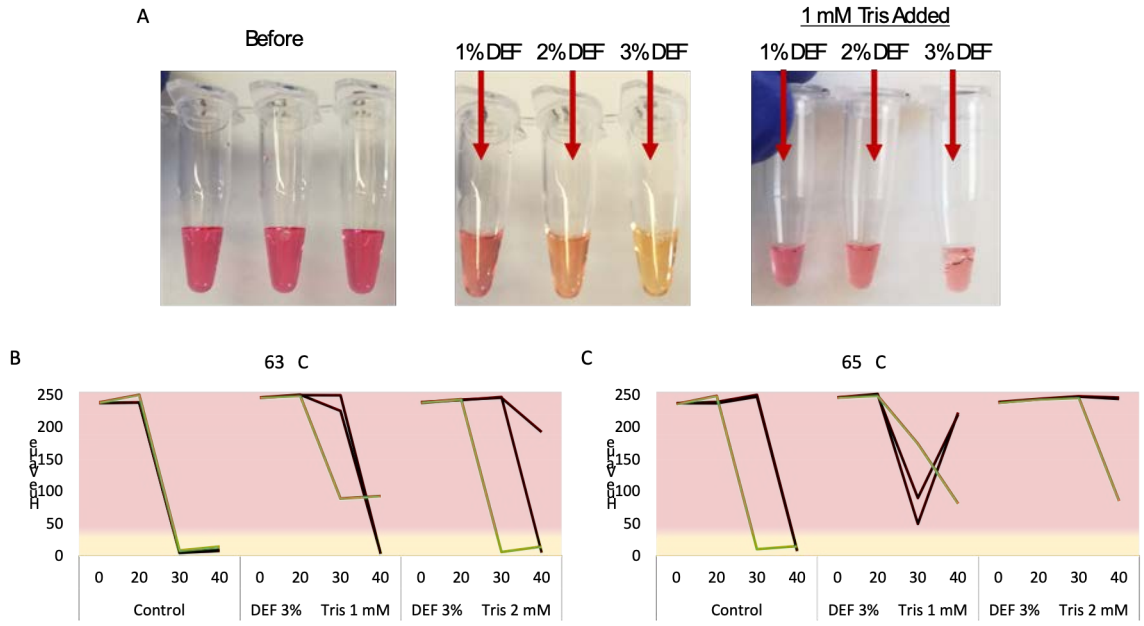

Figure 7 - *Attempt to eliminate non-specific amplification. A. 3% DEF added to LAMP reagents only without sample. B. Vaginal fluid primer set testing with 3% DEF and 2 mM Tris. The control group did not have DEF or Tris added to the reaction.*

3.B.1.d Pre-LAMP DNase Treatment

As discussed above, numerous primer sets specific for both VF and MB target amplification were tested exhaustively with little success, alone or in combination with strategies known to suppress non-specific amplification. This suggested that the primer sets showing off-target annealing with targets in other fluids, were poor candidates for attaining the necessary specificity. However, before abandoning this path, we explored other aspects of the protocol, specifically, alternative lysis and purification methods. We postulated that residual DNA remaining in the purified sample RNA was contributing to off-target annealing and NSA. Hence, we evaluated the effect of an on-column DNase treatment in conjunction with the RNA purification protocol to ensure the samples only contained RNA material. For each primer set, both a DNase-treated sample and a control sample (no DNase treatment) were tested in triplicate, along with duplicated negative controls (water). The results in **Figure 8A** and **Figure 8B** show amplification with VF and MB primer sets in the presence of all body fluids, respectively, at 63 °C for 60 minutes. The right colorimetric profiles in **Figure 8A** (solid lines) show that DNase-treated samples gave target-specific amplification, while the control samples (left panel, dotted lines) showed amplification of other fluid targets with VF and MB primer sets. The post-amplification products analyzed on an electrophoretic microchip (Bioanalyzer) revealed that control samples (no DNase-treatment) exhibited off-target amplification (**Figure 8C**), while those that were DNase-treated were target-specific, as evidenced by the pattern of fragments in Lanes 1 and 7. Since the off-target amplification likely resulted of primer mis-annealing to gDNA,, all samples in future experiments involved DNase-treatment, and the current primer sets for VF and MB were carried forward for further optimization with a goal of 30-minute T_{PR}.

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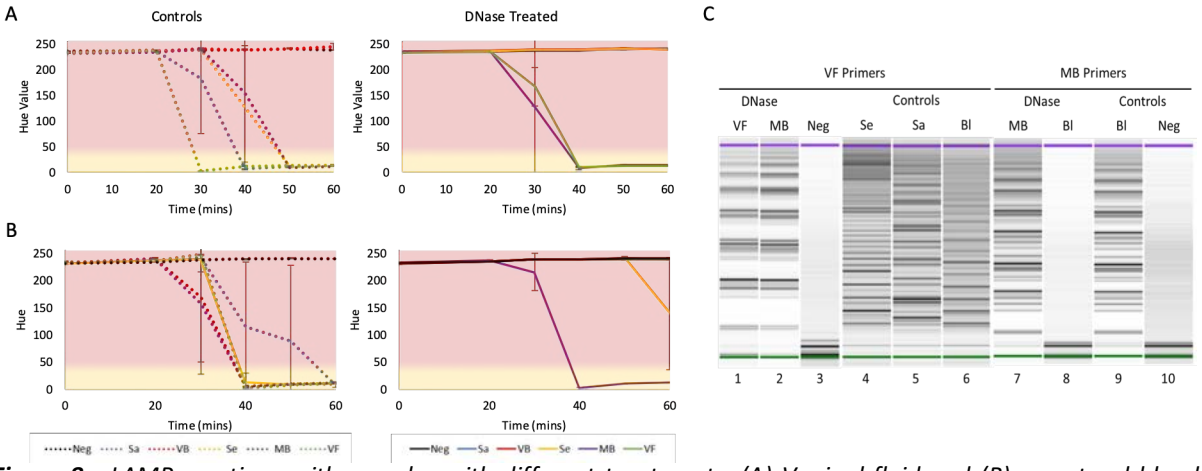

Figure 8 - *LAMP reactions with samples with different treatments. (A) Vaginal fluid and (B) menstrual blood primers amplified at 63°C. The results show amplification of only the targeted fluid in the DNase-treated samples (left panel). The positive range (yellow) is three standard deviations from the average value of the targeted fluid at 60 minutes. The negative range (red) is three standard deviations from the average value of all samples at 0 minutes. (C) Post-LAMP product separated on a microchip-based electrophoresis instrument, Bioanalyzer. Lanes 1-2, 8, 10 show specific, on-target amplification products. Lanes 3 and 10 show a negative control. Lanes 4-6 show off-target amplification products exhibiting random patterning, a result of non-specific amplification.*

Previously, VB, Se, Sa primer sets were optimized for effective isothermal amplification at 63 °C, with the LAMP process complete in 30 minutes. However, the samples used in these studies had undergone RNA purification without a DNase treatment. Hence, the primers for these three fluids were re-tested to evaluate specificity with DNase-treatment and LAMP at 63 °C. These results are given in **Figure 10A**, and show that the target-specific amplification is successful with the DNase-treated samples using the VB primer set and, notably, completed in 20-minutes. Since the blood-based target is beta-hemoglobin (HBB), both VB and MB are expected to amplify. However, as shown in **Figure 8B**, the MB primer set provides a means of discriminating between the two fluids. At 60 minutes, VF and Se samples showed signs of amplification with the VB primer set, but was, unfortunately, confirmed by microchip electrophoresis as NSA. The late onset of these off-target amplifications is a common phenomenon with sensitive primer sets (personal communication), but can be minimized by shortening the assay running time.

Amplification of DNase-treated samples using the Se primer set successfully identified the known semen sample within 40 min, as shown in **Figure 10B**. One VF sample replicate (green solid line) amplified by the 60-minute mark, and was confirmed by the microchip electrophoresis to be amplification that was specific (on-target), suggesting the donor may have had sexual intercourse within 72 hours of sample collection.

Finally, **Figure 10C** shows none of the DNase-treated or control samples know to contain saliva, amplified with the saliva primer set. It is known that saliva has a lower RNA concentration than other fluids[21] due to ribonucleases ubiquitous in the salivary fluid. While this primer set has been shown to be effective in previous experiments, one way to improve efficiency was to increase the amount of sample added to the LAMP reaction. This modification, along with increased polymerase or primer concentrations, provide the path to the enhanced sensitivity and reduced amplification time needed to achieve the targeted assay time of 30 min.

3.B.1.e Saliva Target LAMP Optimization

While the Sa primer set was effective in previous experiments with non-DNase treated samples, DNase-treatment of samples was shown to inhibit amplification. This could be due to the low expression of the Sa target in saliva, in combination with saliva having a lower RNA concentration than other body fluids[21] due to the presence of abundant endogenous RNases. To improve efficiency of target amplification, various approaches were tested in troubleshooting the absence of amplification via RT-LAMP with Sa primers. This included increasing the volume of lysate added, the inclusion of an RNase inhibitor to the LAMP reaction, incorporating a 'protection reagent' directly after sample collection, and/or increasing input volume of neat saliva to the extraction. Even when the RNA sample volume was increased to 25% of the total LAMP reaction (3.12 of 12.5 µl), there was no detectable amplification (**Figure 9A**) over a total RNA mass range of 2.5 to 9.4 ng from two different donors. However, amplification was improved by either the addition of RNase inhibitor, or the incorporation of a 'protection reagent' after sample collection. Going forward, the 'protection reagent' approach may have more utility for long-term storage samples by minimizing degradation. Next, effort was focused on increasing the input volume of neat saliva for the extraction method. This was achieved by increasing the input from 30 µl to 200 µl, and this was effective at for obtaining consistent amplification of the saliva target. **Figure 9B** shows the absence of amplification with 30 µl neat saliva, followed by inconsistent amplification of replicates with 60 µl of neat saliva, but highly consistent amplification with 200 µl

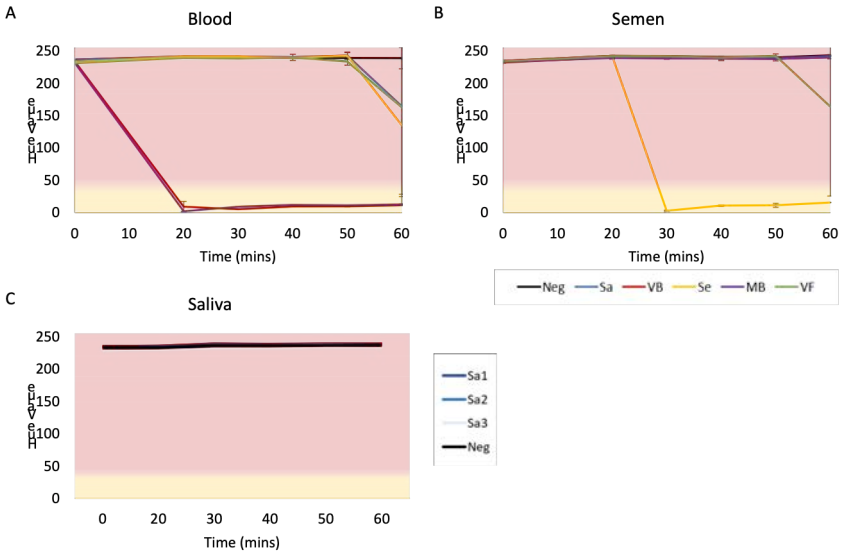

Figure 10 *- Specificity test using DNase-treated samples in LAMP at 63°C with Blood (A), Semen (B), and Saliva (C) primer sets. The results show amplification of only the target fluid in the VB and Se DNase-treated samples. The Sa primer set was tested with three DNase-treated donors and showed no amplification. The positive range (yellow) is three standard deviations from the average value of the target fluid samples at 60 minutes. The negative range (red) is three standard deviations from the average value of all samples at 0 minutes.*

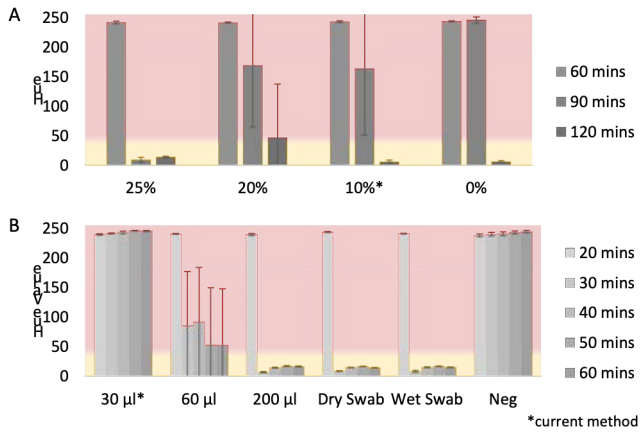

Figure 9 *- Various approaches to increase amplification of Sa RNA target. (A) By increasing the volume of purified RNA sample to the LAMP reaction, amplification was achieved, but at a slower rate. (B) The input of 200 µl of neat Sa or buccal swab showed consistent amplification of the Sa RNA target. Each bar represents the standard deviation of triplicate RT-LAMP from three donors.*

in 30 minutes. Successful amplification was also observed for whole dry or wet buccal swabs. The data was compiled from RNA extractions from three donors indicating low variability in the concentration of the RNA target from donor-to-donor at these sample volumes. In addition, no NSA was detected after 60-minutes of LAMP.

Having solved the saliva amplification problem, the ability for specific target amplification of all five fluids within 30 minutes was finally achieved. To achieve sensitivities similar to, or better than, methods described in the current literature, we sought to determine if the LAMP method would be effective with low concentration samples.[22-25] Using 200 µl of neat saliva in the purification method, optimization of specificity and sensitivity of the LAMP assay was completed (**Figure 11**). For specificity, the saliva primer set was tested against all five body fluids used in this research. For sensitivity, the RNA lysates were quantified using RiboGreen™ on a Nanodrop 3300 and diluted to various concentrations of Total RNA. This primer set was specific for saliva, with a lower limit of detection of 6.25 ng of total RNA. While this primer set was associated with a lower sensitivity than we had hoped for, the sensitivity is, surprisingly, comparable to other research methods.[26-28] The common presumptive Sa methods (α-Amylase or Lateral Flow Assay) used in forensics have sensitivities at approximately 1:100 dilution[22], but these methods detect enzymes or proteins, which also have been identified in other fluids. Thus, we believe that continued effort on this front is justified, as providing a novel and more specific saliva identification test would be valuable.

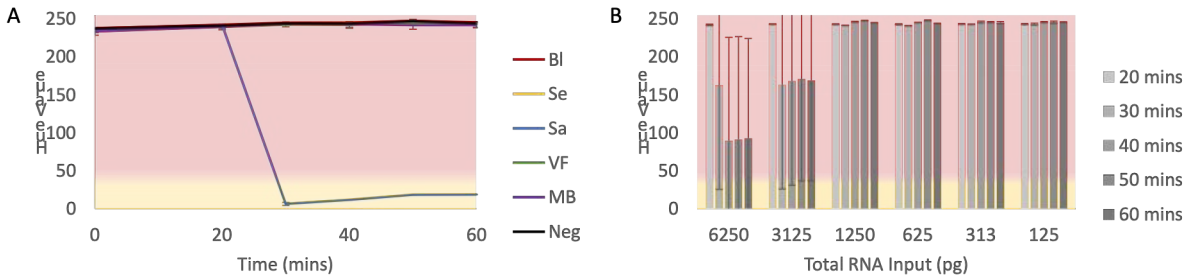

Figure 11 - *Specificity and sensitivity of saliva RNA target. (A) Known fluids were amplified against the saliva target for specificity testing. (B) Known salivary fluid was quantified via UV Nanodrop and diluted for amplification. After 60 minutes of LAMP, 2 of 3 replicates amplified at 6.25 ng. Each line/bar in graphs represents an n=3 from one donor.*

As a result of the LAMP amplification of the other four body fluids being effective, we sought to define the sensitivity for those fluids. These amplifications were carried out at the optimized 63 °C in a Veriti thermal cycler. **Figure 12** shows the VB primer set has high sensitivity after 30 mins with ~31 pg of Total RNA amplifying effectively. Currently, blood is detected via a phenolphthalein tetramethylbenzidine (PTMB) (i.e., tests for peroxidase-like activity ideally of hemoglobin) or protein lateral flow assay (e.g., human glycophorin A) with variable sensitivity of roughly 1:1,000,000 dilution of whole blood[29, 30] for both assays. While not a direct comparison, it is noteworthy that the LAMP results are based on RNA extraction and purification from only 10 µl of whole blood.

In forensics, seminal fluid and/or semen is presumptively detected using either an enzyme test (e.g., acid phosphate) or protein lateral flow assay (e.g. semenogelin, PSA), with confirmatory testing by 'Christmas Tree' staining cells from a sample. The staining uses kernechtrot and picroindigocarmine to stain the epithelial cells green and acrosomal cap of the sperm heads red/pink, respectively[31]. With microscopy, if a single sperm cell is identified, the evidence is carried forward for DNA profiling. **Figure 12** shows that LAMP was able to detect approximately 300 pg of Total RNA after 40 minutes of amplification. For comparison, the sensitivities of the lateral flow assay and enzyme test are ~2.5 nL of human semen[32] and 1:200 dilution of human semen[33], respectively. Similarly, vaginal fluid and menstrual blood were also tested for sensitivity using the primer set that provided the best specifically. The LAMP approach allowed for both of these fluids to be detected at ~300 pg of Total RNA after 30 minutes. Unfortunately, there is no presumptive or confirmatory test currently used in forensic science to compare with this assay. However, the literature describes research endeavors that show sensitivity similar to these values.[26, 34-36] Finally, all of these values are taken from a single donor, thus at least two other donors will be tested with all five fluids to show the expression of these targets among donors and, hopefully, similar sensitivities in the LAMP assay.

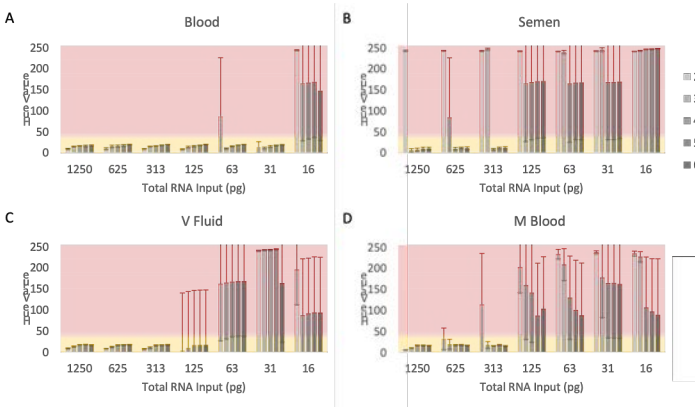

Figure 12 - *Sensitivity of four forensic body fluid's mRNA LAMP targets. Each line/bar in graphs represents an n=3 from one donor.*

3.B.2 BODY FLUID ASSAY TESTING WITH MOCK SAMPLES

To further evaluate the capabilities of the LAMP approach, forensically-relevant mock samples were prepared and analyzed in a 'single-blind study'[1]. **Table 2** shows the results of the mock sample analysis, where the fluids deposited on the swab for each sample is identified in the table by the shaded the cell. The mock samples consisted of mixtures up to three body fluids with volumes at: 5 µl Blood (Bl), 10-20 µl Sa, 2 µl Semen (Se), 5 µl menstrual blood (MB), or whole vaginal fluid (VF) swab. They were allowed to dry at ambient temperature overnight, extracted the following day, with roughly one third of each swab used in an extraction process that incorporated on-column DNase treatment. The RT-LAMP reactions were incubated in a thermal cycler at 63 °C for 60 minutes, with images captured (manually) at 0, 20, 30, 40, 50 and 60 minutes. The images were analyzed via Image J and the data interpreted by another scientist who had no knowledge of the make-up of the samples. The results show that two of the five mock samples were correctly identified (**Table 2**). In addition, all of the negative controls were correctly analyzed as having no amplification.

[1] Single blind study: first scientist made mock samples; second scientist analyzed the samples without knowledge of the true nature of the samples.

Table 2 - *Colorimetric results from prepared mock samples. Of the five mock samples, two samples were called overall correct. The other three mock samples failed due Sa levels lower than sensitivity levels and/or false presence of MB. If the two replicates were not the same result, the mock sample was concluded as inconclusive.*

Mock Sample # & Read-Out	Blood	Semen	Saliva	MB	VF	Correctness by Sample
1	Correct	Correct	Incorrect	Inconc	Correct	70%
2	Correct	Correct	Correct	Correct	Correct	100%
3	Correct	Correct	Correct	Correct	Inconc	90%
4	Incorrect	Correct	Incorrect	Incorrect	Correct	40%
5	Inconc	Correct	Inconc	Incorrect	Correct	60%
Correctness by Body Fluid	70%	100%	50%	50%	90%	

The incorrectly identification of the three samples was linked to the presence of saliva and menstrual blood. The volume of saliva deposited onto the swabs likely contains a mass of RNA that is below the limit of detection discussed in the previous section; hence, the saliva target was not expected to amplify. The volume of neat saliva chosen was guided by mock samples described in the literature specifically defined to mimic volumes typically collected at a crime scene.[37-39] With the analysis of the next set of mock samples, a higher volume of neat saliva was used to show the target is capable of amplification in a mixed body fluid sample.

The most significant mis-identification seemed to center around the MB primer set. Of the three incorrectly-called mock samples, the MB target was identified as either 'inconclusive' or 'positive' in every sample. Due to the nature of the fluid, not surprisingly, VF co-amplifies with MB. Mock sample #1 resulted in amplification of the MB target in one of the two replicates after two rounds of testing. This points to the possibility that there was a low concentration of the menstrual blood target in the sample. The target for the MB primer set (matrix metallopeptidase 10: MMP10) functions to breakdown extracellular matrix, and is highly concentrated in the endometrium[i]. However, MMP10 may be present in low concentrations in other body fluids[36] and, hence, has the potential to lead to false positives. When assessing the results of mock samples #4 and #5, both samples contained VF and were positive for MB. Both VF and MB are from the same anatomical area, which means a positive VF result is expected when a sample contains MB. However, when only VF was present, MB target amplification should not occur. Since the identity of body fluids deposited on each mock sample was known, the results indicate that MMP10, indeed, may be present in VF samples.

3.B.2.a Troubleshooting Menstrual Blood Target Detection

The basis for MB target amplification in a VF sample without visual discoloration was puzzling. One explanation could be the availability of newer forms of contraception that change the menstrual cycle. Historically, birth control pills were the popular choice of contraception for women, which allowed for one menstrual cycle per month. Newer contraceptive approaches allow for cycles every few months or no cycle at all, depending on the type of device. This presents the possibility that the MB target is secreted without the visual color of the fluid because the body may discard any extracellular matrix to prevent the menstrual cycle from initiating. To provide evidence for this, freshly donated VF samples (n=5, outside menstrual cycle) were

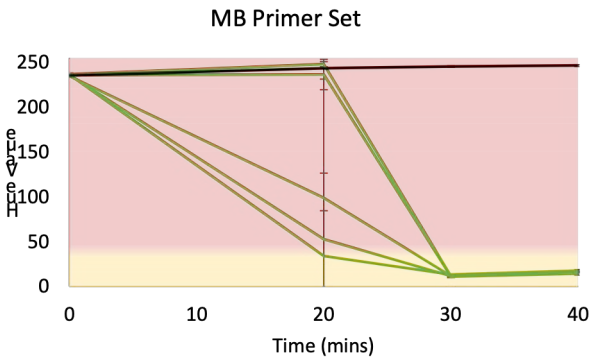

Figure 13 *- Amplification of the menstrual blood target. To determine if the MB target is present, known VF samples were amplified. All of the samples amplified with a hue increase from the negative control (black) hue value. Each line represents an n=3 from five donors.*

extracted with on-column DNase treatment. The swabs did not show any evidence of red discoloration indicative of blood. Following extraction, the samples were LAMP amplified with the VF primers, and then with the MB primer set (**Figure 13**). All VF samples amplified with the MB primers in 30 minutes, indicating the presence of MMP10 in the samples.

In addition, the eight primer sets targeting MMP10 and MMP11 previously used for MB were tested, which all showed amplification with VF-only samples. **Table 3** shows that some primer sets led to NSA in negative controls, while other led to off-target amplification with other body fluids. In short, none of these primer sets exhibited the specificity needed for MB identification. However, having an extra primer set that amplifies VF samples could serve as a secondary target for VF, or alternatively serve to discriminate between VB and MB. After discussion with a number of forensic analysts, it became clear that the need to discriminate VB and MB was not a high priority in most forensic cases. Since the VB primers amplify both VB and MB, we decided to discontinue testing with MB primers. However, for now we will have the menstrual blood target as an optional LAMP test and continue further testing with the body fluid panel for venous blood, semen, vaginal fluid, and saliva.

Table 3 *- Menstrual blood primer specificity. Multiple targets were assessed to identify a specific primer set for MB. Across multiple samples the following primer sets were deemed to show high non-specific annealing via primer-dimers or off-target annealing in other body fluids. MB = menstrual blood, VB = venous blood.*

mRNA	Primer Set	Loop Set	MB	VB	Neg	Conclusion
MMP11	ID 4	2	30 min	Amped VF	60 min	NSA
		54	60 min	60 min	45 min	NSA
	ID 98	2	40 min	40 min	40 min	NSA
		9	30 min	30 min	50 min	NSA?
MMP10	ID 47	2	45 min	45 min	None	Not Specific
		6	60 min	45 min	None	Not Specific
	ID 100	8	30 min	30 min	None	Not Specific
		308	45 min	30 min	None	Not Specific
		250	20 min	Amped VF	None	Not Specific
LEFTY2	ID9	311	30 mins	35 mins	None	Not Specific
		3	30 mins	40 mins	None	Not Specific
		11	30 mins	40 mins	None	Not Specific
		78	30 mins	40 mins	None	Not Specific

3.B.2.b Assay Testing with Single-Blind Mock Samples

The goal of this study was to assess specificity of our final protocol using twenty mock samples that best imitate crime scene samples. The samples were prepared by depositing a range of body fluids at various concentrations, as single source or mixtures, onto cotton swabs, cloth, or denim (Error! Reference source not found.). The deposited fluids were not limited to the body fluid panel described thus far, but also included breast milk and nasal mucus. The samples were dried overnight or aged up to 5 years at room temperature (RT) without light. Approximately ¼ of the

swab (or similar-sized cutting for samples on fabric) was cut, sealed in a tube, and assigned a sample ID before transferring it to a scientist for processing and analysis; the scientist was blind to the identity of the sample. The mock samples were amplified for detection of each body fluid target using the optimized assay conditions and, when necessary, a confirmatory test was performed. The confirmatory test was either a microchip electrophoresis or a PSA lateral flow assay for Se. The LAMP assay was performed at 63 °C in the Veriti thermal cycler and in the integrated system (iLAMP).

To our disappointment, this study correctly identified only seven of the 20 mock samples; we have a few postulates as to why the outcome was poor. Assays targeting Sa and VF performed the best when tested with all of the mock samples in both the Veriti and iLAMP (**Table 4**). The Sa assay amplified all of the mock samples correctly, and the VF target amplified 90% (18/20) of the mock samples correctly in the Veriti. The two samples misidentified in the VF assay consisted of female nasal swab with male saliva, and breast milk with male saliva. It has been shown that low concentrations of VF target can be found in saliva, providing a potential reason for the false positive identification.[40, 41] With iLAMP, the Sa assay misidentified three samples (80%) while the VF assay misidentified only one (93%). Even though there are misidentifications, the results show promise for future use in a forensic assay.

Table 4 - *Hue analysis of mock samples amplified in the Veriti (TC; thermal cycler) and Integrated system (iLAMP). The VB results were taken after 40 minutes and the Se, Sa, VF results were taken after 60 minutes of isothermal LAMP. Confirmatory tests were performed on VB (Bioanalyzer) and Se (PSA Test). Each red box denotes an incorrect identification.*

Sample Number	Blood			Semen			Saliva		V Fluid		Overall Conclusion (Veriti)
	TC	iLAMP	Bioanalyzer	TC	iLAMP	PSA Test	TC	iLAMP	TC	iLAMP	
B001	Neg	Neg		Neg	Neg	Pos	Neg	Pos	Pos	Pos	Incorrect
B002	Pos		TRUE	Neg		Pos	Neg		Pos		Incorrect
B003	Pos	Pos		Pos	Neg		Neg	Pos	Pos	Pos	Correct
B004	Pos	Pos		Neg	Neg		Neg	Neg	Pos	Pos	Correct
B005	Pos	Pos	TRUE	Neg	Neg		Pos	Pos	Pos	Neg	Incorrect
B006	Neg	Neg		Neg	Neg		Pos	Pos	Pos	Pos	Correct
B007	Pos			Neg			Neg		Neg		Correct
B008	Pos	Pos		Neg	Neg		Neg	Neg	Neg	Neg	Correct
B009	Neg	Pos	TRUE	Neg	Neg	Pos	Neg	Neg	Pos	Pos	Incorrect
B010	Pos		TRUE	Neg			Pos		Pos		Incorrect
B011	Pos	Pos		Neg	Neg		Neg	Neg	Neg	Neg	Correct
B012	Neg	Neg	NS	Neg	Neg	Pos	Neg	Pos	Neg	Neg	Incorrect
B013	Neg	Neg	NS	Neg	Neg		Neg	Neg	Neg	Neg	Correct
B014	Neg	Pos	NS	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Correct
B015	Neg		NS	Neg		Neg	Neg		Pos		Incorrect
B016	Neg	Neg	NS	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Incorrect
B017	Pos		TRUE	Neg		Neg	Neg		Pos		Incorrect
B020	Neg	Neg		Neg	Neg	Neg	Neg	Neg	Neg	Neg	Correct
B021	Neg	Neg		Neg	Neg		Pos	Pos	Pos	Pos	Incorrect
B022	Neg	Pos	TRUE	Pos	Neg		Neg	Neg	Pos	Pos	Correct
Correct Identification	80%	73%		65%	53%		100%	80%	90%	93%	50%

Previous testing showed that both Se and VB assays had great specificity and sensitivity with samples from various donors. However, the performance of these assays (**Table 4**) was

unexpected. For the Se assay, seven mock samples did not amplify in the Veriti (35%) or iLAMP (47%). After the sample identities were revealed, it became clear that inclusion of samples that have been aged over years (B012), on denim (B009), or have PCI >24 hours, could play into the false negative results. The false negative samples were analyzed with a commercial lateral flow assay for the presence of PSA; the test confirmed the presence of PSA in all samples, except the PCI samples and breast milk. However, the LAMP Se assay was able to detect the target in the PCI 24hr sample, where the PSA test did not. The VB assay performed comparably in the two systems, both showing 'positive' results with samples devoid of blood. To troubleshoot this, amplified samples from the Veriti were microchip electrophoresed to determine if the amplified fragments were specific for the blood target or the result of NSA. This confirmed that the VB primer set is amplifying the target in a sample where the VB target is not present; it is possible that the reagents are contaminated from the extraction kit or the LAMP assay.

Overall, the mock sample study showed which primer sets performed well and which need further optimization. The Sa and VF primer sets worked effectively in both systems (>80%), the VB primer set performed moderately (>70%), and the Se primer set performed the worst (>50%). The Se primer set underwent further analysis that showed the LAMP assay was not as sensitive as the PSA test, but could amplify target in PCI samples. Due to the lower than expected results from the VB assay, we focused on troubleshooting the cause of this.

Table 5 *- Hue analysis of the VB target in the mock samples at various temperatures. The percentage of incorrect calls increased with increased temperature, which was not hypothesized.*

	Thermal Cycler		
Sample	63 C	65 C	67 C
B001	Neg	Neg	Pos
B002	Pos	Pos	Pos
B003	Pos	Pos	Pos
B004	Pos	Pos	Pos
B005	Pos	Pos	Pos
B006	Neg	Neg	Pos
B007	Pos	Pos	Pos
B008	Pos	Pos	Pos
B009	Neg	Pos	Neg
B010	Pos	Pos	Neg
B011	Pos	Pos	Pos
B012	Neg	Neg	Neg
B013	Neg	Neg	Neg
B014	Neg	Neg	Neg
B015	Neg	Neg	Neg
B016	Neg	Neg	Neg
B017	Pos	Pos	Pos
B020	Neg	Neg	Neg
B021	Neg	Neg	Neg
B022	Neg	Pos	Pos
Incorrect Calls	20%	30%	30%

3.B.2.c Troubleshooting Data from the Mock Study

The first troubleshooting test involved increasing the LAMP assay temperature, which should decrease the probability of NSA by reducing mismatched primer binding to the primers themselves or similar non-primer sequences. While the previous results do seem to point to contamination, the microchip electrophoresis results showed significant amount of NSA that could potentially be eliminated with assay higher temperatures. All of the mock samples were amplified with the VB primer set at 63, 65, and 67 °C, and the results of hue image analysis at the 40-minute time point is given in (**Table 5**). Unfortunately, the overall success decreased as the temperature increased. Of the 20 mock samples amplified, eight yielded false results at 65 °C or 67 °C. Of the previous four samples that yielded false results at 63 °C, three also were mis-called at both higher temperatures, showing no positive effect with increased temperature. Sample B010 did amplify incorrectly at 65 °C, but did not amplify at all at 63 °C or 67 °C. Samples B001, B006, B009, and B022 all amplified incorrectly at the higher temperatures, but did not amplify at 63 °C. Since these results failed to clarify why the primer set or the mock samples amplified, we tested reagent blanks and blank swab samples to determine if contamination could be the issue.

This allowed us to identify and test various possible contamination points throughout the bfID process (e.g., reagents in RNeasy Mini kit, swabs, LAMP reagents).

3.B.3 Y-SCREENING ASSAY DEVELOPMENT

A DNA-based Y-screening approach was developed as an alternative to immunological-based p30 assay for sperm detection. Early phase of research focused on a DNA lysis method, which is followed by efforts on the LAMP assay targeting human male DNA. The final phase of research focused on a thorough evaluation of the Y-screening approach with dilution studies, mock sample analysis, and comparison with conventional p30 assay.

3.B.3.a Optimization of sample lysis

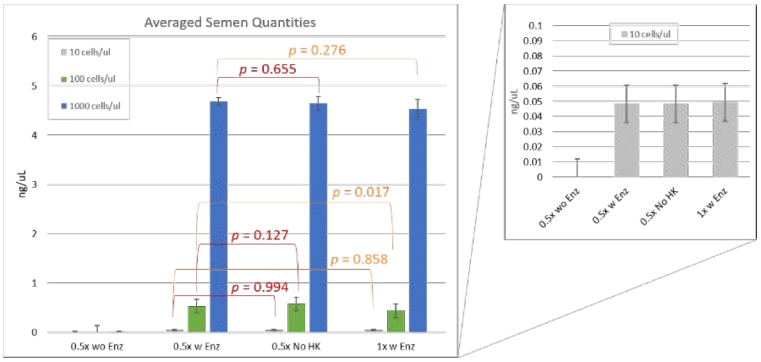

Figure 14 *- Optimization of sperm lysis. Three concentrations of sperm cells from neat semen were lysed in four conditions. The "0.5x w Enz" is the final optimized condition used in further experiments.*

While the application of Y-screening is not limited to sexual assault kit (SAK) analysis, we chose to work with neat semen as the substrate for lysis optimization to ensure the method is effective against the most resilient cell type. The lysis method relies on an augmented commercialized enzyme-based kit[2] to efficiently release amplification-ready DNA in a closed-tube format in 9 minutes. With considerations for the phenol red LAMP chemistry, which limits 2 mM Tris buffer carryover from lysate, the buffer supplied with the kit was halved in the reaction to keep compatibility. As shown in **Figure 14**, the lysis efficiency at "0.5x w Enz" is statistically no different than "1x w Enz" across three different semen concentrations tested. Also shown in **Figure 14** is "0.5x wo Enz", which was a control group to show that in the absence of the enzyme, negligible amount of DNA was detected, suggesting intact sperm cells were used. The lysate from this protocol was shown to be compatible with both NEB's Colorimetric LAMP kit (contains phenol red) as well as an in-house LAMP mix using NEB's *Bst* 2.0 WS polymerase (contains hydroxynaphthol blue, HNB). The lysis method was further evaluated using a common forensic sample acceptor – cotton swabs – which did not interfere in obtaining amplifiable DNA via LAMP. This concluded the optimization for step one of the Y-screen process.

3.B.3.b Optimization of the Y-screening LAMP assay

The second phase of development involved the optimization and characterization of the LAMP assay targeting human male DNA. Using a published study by Nogami et al. as the reference point[1], we demonstrated improved assay speed by re-designing primers. Still targeting Y-amelogenin, primer ID50-82 contains a full pair of loop primers, which has shown to accelerate DNA amplification by providing additional annealing and extension sites [42]. The accumulation of DNA product in LAMP is accompanied by an increase in turbidity of the reaction, which serves as

[2] ZyGEM SexCrime kit

the detection modality used by Nogami et al. To aid visual and digital evaluation of the reaction, we implemented colorimetric indicators as mentioned previously. The color change of HNB and PR can therefore be monitored and quantified objectively, removing variability in human color perception and bias associated with interpreting p30 assays. The objective analysis workflow is shown in **Figure 15**. Equipped with uniform, diffused lighting in the imaging box, samples were captured with a Huawei P9 smartphone at time points to document the color change. The region of interest (ROI) for each sample can be selected and converted to Hue component for analysis. The resulting columns graph showed clear distinction between a negative and positive value. The same analysis can be performed with PR.

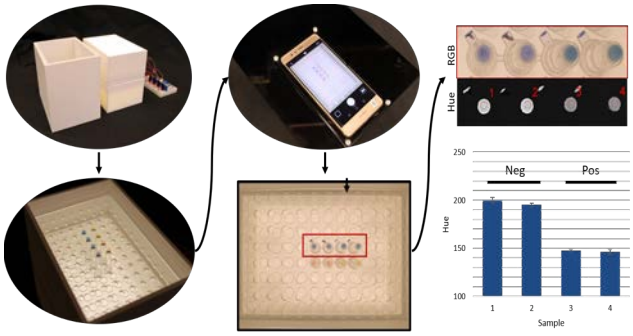

Figure 15 *- Implementation of Hue measurements in colorimetric LAMP reactions. An imaging box equipped with diffused white light allowed a 96-well plate to be imaged by a smartphone. The RGB color image can then be converted to greyscale hue for analysis. The Hue values within the circular ROIs were graphed to show a clear distinction between a positive and negative reaction. Error bars in column graph show standard deviation of Hue within the ROI.*

Early in our studies, male-specific LAMP primers were determined to be specific to human male DNA. After extensive testing, however, non-template amplification (NTA) was observed occasionally in NTC. The occurrence of NTA in LAMP is due to several factors, including the numerous primers used at high concentrations, and reaction conditions such as high magnesium concentration [43]. We explored a number of approaches for improving specificity such as the addition of additives (e.g., betaine or diethyl formamide), but ultimately it was increasing the assay temperature from 63 to 65°C that found to be most effective.

3.B.3.c Dilution studies for the evaluation of the Y-screen protocol

After completing LAMP assay optimization, a dilution study was performed to evaluate the Y-screen protocol as a unit. Neat semen was used undiluted or serially diluted to 1:2, 1:10, 1:100, and 1:1000 before deposited onto cotton swabs. Female buccal cells were collected directly onto cotton swabs as a negative control. A quarter of the swab was cut and underwent cell lysis in triplicate, then each lysate was amplified by LAMP in duplicate. **Figure 16** shows colorimetric LAMP results in a semi-real time manner. Recall that a high hue value is negative(no amplicons), but as LAMP proceeds successfully generating amplified products (a positive), the hue value decreases. The Pos showed a color change pass the threshold by 30 min, that is, the time to positive (Tp) value was 30 min. NTC and female samples (F) remained negative, indicating specificity. Regardless of the dilution factor, swabs containing semen had a Tp around 30 or 40 min. Next, qPCR was performed with Plexor HY System.

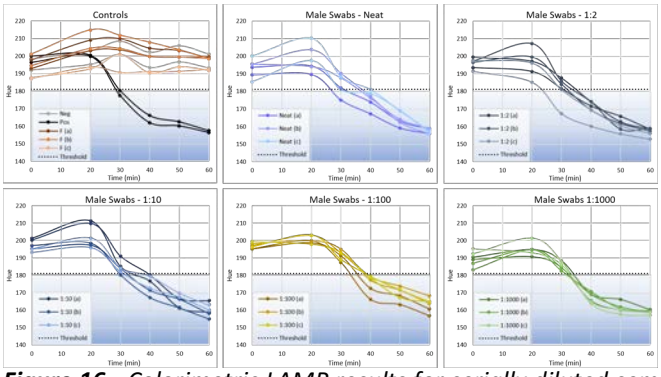

Figure 16 *- Colorimetric LAMP results for serially diluted semen lysate. Hue value (Y-axis) for each sample was analyzed at various timepoints (X-axis). A hue value below the threshold (dotted black line) into the blue shaded zone indicates a positive reaction.*

Figure 17, autosomal DNA concentration is shown in green, and male (Y) DNA is shown in blue. Plexor confirmed the absence of Y DNA in the female samples, which corroborates Y-screen results. The lowest average autosomal DNA concentration was 0.132 ng/µL in the 1:1000 sample, whereas the highest was 10.526 ng/µL in the 1:2 sample. These approximates to 40 to 3000 cells/µL, respectively. The lysis method yielded DNA concentrations that followed a linear correlation with cell dilutions from 1:1000 to 1:10. Higher cell numbers plateaued in DNA concentration potentially due to limited lysis time. Internal positive control (IPC) revealed no flag in any of the samples, suggesting no inhibition was detected.

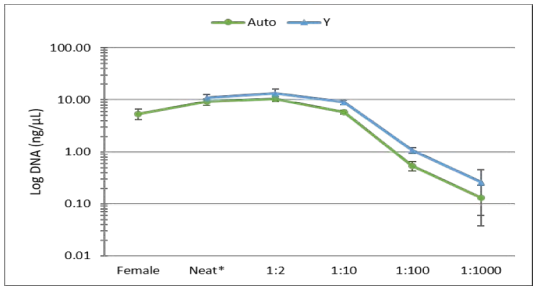

Figure 17 *- DNA quantification by Plexor HY System. The autosomal concentration is shown in green and Y concentration shown in blue. DNA concentration is shown in logarithmic ten scale. Error bars show standard deviation of three replicate.*

The final step in forensic DNA analysis is the generation of a CODIS-eligible profile. Therefore, correlation between Y-screen and PowerPlex Fusion results were made using the same lysate from step one of Y-screen. Complete profiles were obtained from all samples, and the peak height (PH) from 24 loci are shown in **Figure 18A,** with input template mass graphed on the secondary Y-axis. Unsurprisingly, the most dilute samples produced the lowest average peak height. According to PowerPlex Fusion Technical Manual, 0.25 – 0.5 ng of template DNA in a 25 µL reaction volume is recommended for the optimal peak balance when purified DNA is used [44]. The 1:1000 triplicate ranged in DNA concentration from 0.083 to 0.205 ng/µL, which when added 1 µL to the reaction were outside of recommended amount, and just below the 0.1 ng minimum required DNA. Nevertheless, the use of crude lysate at a sub-optimal DNA amount in PowerPlex Fusion allowed the generation of full profiles. This suggests compatibility of the lysate as well as the tolerance of the STR chemistry. Finally, **Figure 18B** shows the comparison between the percent success rate of PowerPlex Fusion and Y-screen graphed in columns, as well as DNA mass graphed in lines. In summary, Y-screen identified all samples that contained male DNA and gave no false positive in female-only samples (as evidenced in **Figure 18C**). A profile from the 1:1000 dilution sample is shown in **Figure 18D** with all 24 loci present. Here, the lack of inhibition in Plexor analysis and full STR profiles are further evidence that lysates from Y-screen are compatible for

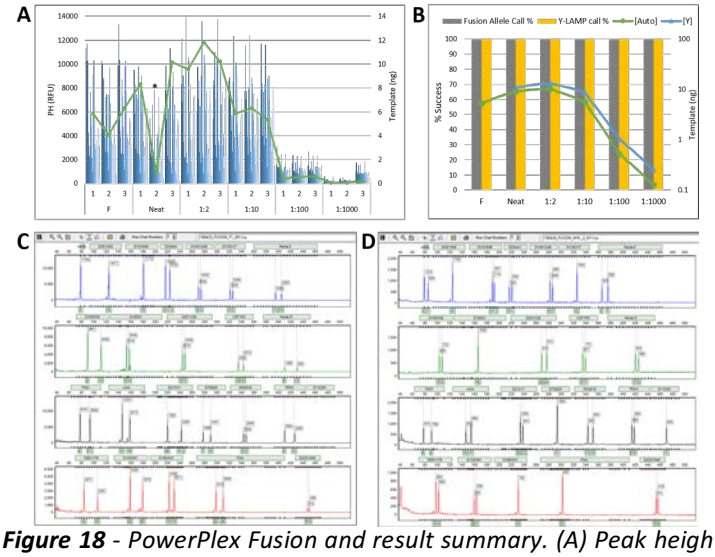

Figure 18 *- PowerPlex Fusion and result summary. (A) Peak height from 24 loci was shown for each triplicate sample per dilution. The * denotes the replicate that was excluded from the Plexor data. (B) PowerPlex Fusion and Y-screen success rates are shown in grey and yellow columns, respectively. Input template is graphed on the second axis (logarithmic ten) in lines. (C) Exemplary profile from female-only sample. (D) Exemplary profile from 1:1000 sample.*

commercialized and validated assays downstream.

To further investigate the limitations of the Y-screen assay, the dilution study was repeated, this time with two additional semen donations to address the wide variation of donor-to-donor cell count (20,000 – 200,000 cells/µL)[45]. Using a hemocytometer, the two donations were estimated to have 91,000 (Donor B) and 41,000 (Donor C) cells/µL respectively. In addition, a serial dilution (to a factor up to 10,000-fold) was performed to mimic casework samples with trace semen. Neat semen samples were serially diluted to 1:100, 1:1000, 1:2000, 1:5000, and 1:10000 before depositing onto cotton swabs. Similarly, a quarter of the swab was cut and underwent cell lysis in triplicate, then each lysate was amplified by LAMP in duplicate. Results of LAMP control samples are shown in **Figure 20A**, and the dilution series are shown in **Figure 20 B-F**. At dilutions 1:100, 1:1000, and 1:2000, all replicates gave positive results. At 1:5000 and 1:10000, however, most samples no longer amplified. For the 1:5000 or 1:10000 samples that did amplify ("5"-1" and "10k-1" swab cuttings), it was inconsistent between LAMP duplicate, suggesting the assay was approaching its detection limit.

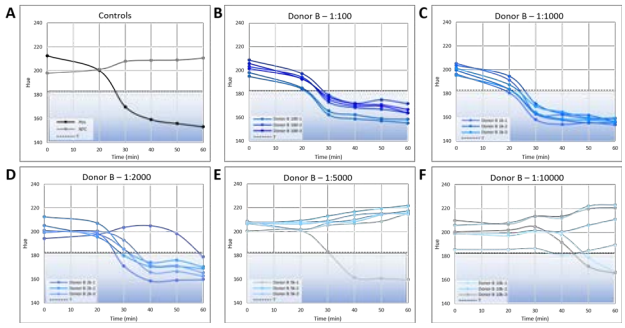

Figure 20 - *Colorimetric LAMP results for serially diluted Donor B semen lysate. (A) Pos and NTC samples. (B) 1:100. (C) 1:1000. (D) 1:2000. (E) 1:5000. (F) 1:10000.*

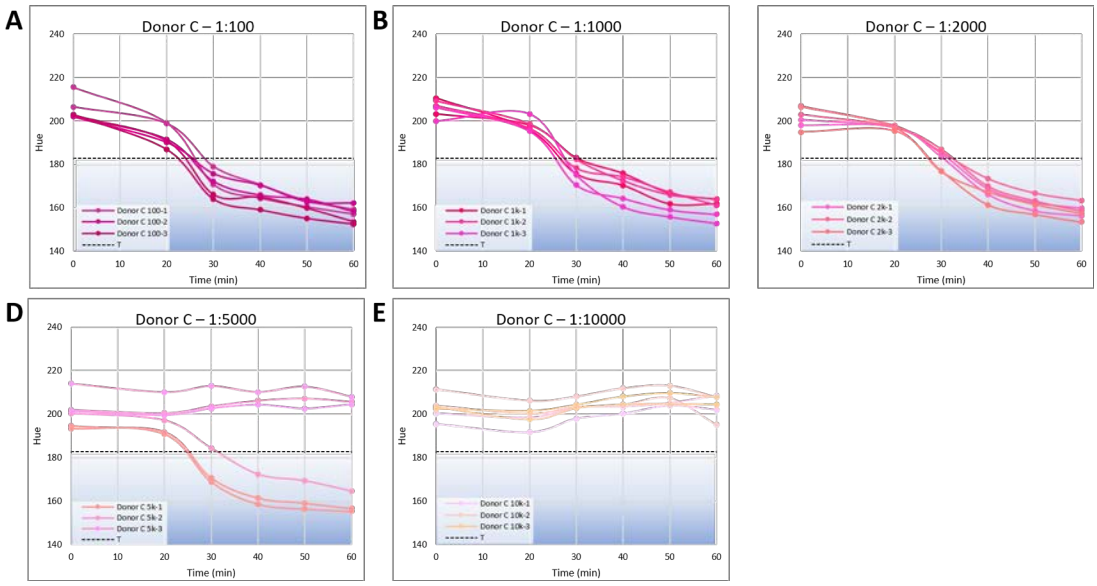

Figure 19 - *Colorimetric LAMP results for serially diluted Donor C semen lysate. (A) 1:100. (B) 1:1000. (C) 1:2000. (D) 1:5000. (E) 1:10000.*

LAMP reactions of serially diluted semen from donor C are shown in **Figure 19A-E**. At 1:100, 1:1000, and 1:2000 dilutions, all replicates amplified had a Tp of 30-40 min. At 1:5000, both "5k-1" samples amplified, and neither "5k-3" samples did. For "5k-2", there was inconsistency in LAMP duplicate. None of the 1:10000 samples amplified. From these two donors, the limitations of Y-screen can be drawn at around 1:5000 semen dilution as indicated by the lack of reproducible amplifications within a lysate from one swab cutting (e.g., Donor C "5k-2") and between replicate swab cuttings (e.g., Donor C "5k-1", "5k-2", and "5k-3" samples).

DNA concentrations were estimated using Plexor HY system. **Figure 21,** now in picograms per microliter in the Y-axis, shows that DNA yield was dilution factor-dependent, and confirms that Donor B gave approximately double the DNA yield in the 1:100 dilution than Donor C due to higher sperm count. However, at higher dilution factors, the yields were comparable, which corroborates with the LAMP results where amplification rates were also comparable. Adding 2 µL lysate to each LAMP reaction, it was equivalent to adding 5.2 and 4.3 sperm cells for Donor B and C, respectively. The extremely low copy number explains the sporadic or lack of amplification at this dilution factor. Finally, similar with the previous dilution study, no sign of inhibition was detected according to the IPC status.

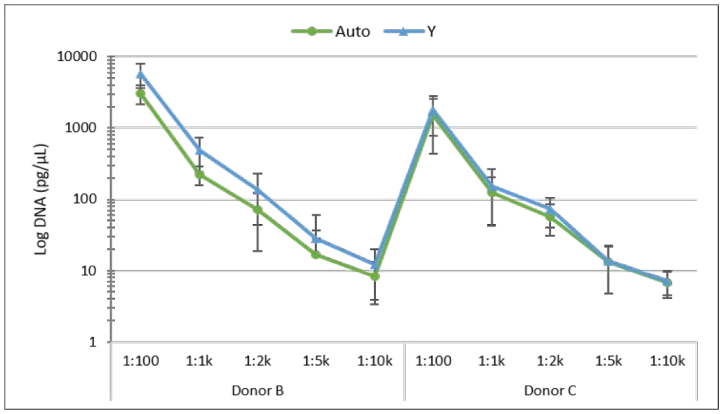

Figure 21 - *DNA quantification by Plexor HY System. The autosomal concentration is shown in green and Y concentration shown in blue. DNA concentration is shown in logarithmic ten scale. Error bars show standard deviation of three replicate.*

Unlike the previous dilution study, this data set was more complex in comparison. The combined data from Fusion profiling, Plexor quantification, and LAMP results are shown in **Figure 22**. Fusion analysis for samples from 1:100 and 1:1000 dilutions was not performed, based on the assumption that full profiles would be produced with ample DNA yield. The top panel displays the PH of each called peak in Fusion in columns (24 total if full profile) with input template on the second

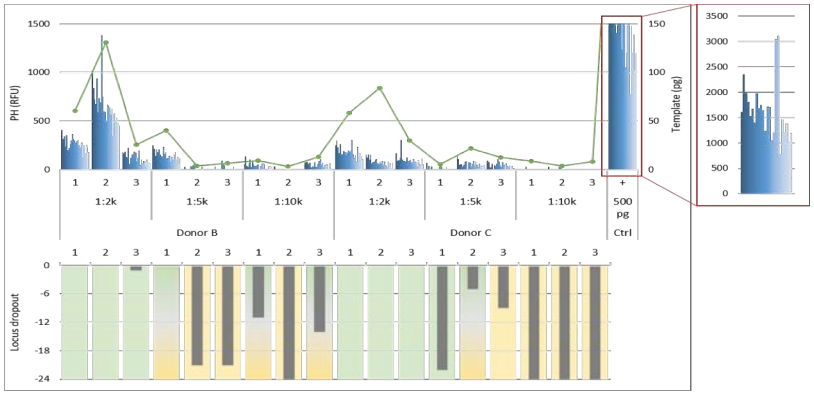

Figure 22 - *Compiled data from PowerPlex Fusion, Plexor HY System, and Y-screen. Top graph shows peak height from 24 loci in columns, and input template in green line. The inset shows peak heights from 500 pg 2800M as positive control. The bottom graph shows number of locus dropout in grey columns. Y-screen results are shown as overlaid shades. Solid green indicates +/+, solid yellow indicates -/-, and gradient green-yellow indicates +/- result in LAMP.*

This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

axis in line. A positive control was performed with 2800M, which is shown in inset on right with the expected PH with 500 pg input template. The lower half of **Figure 22** shows the number of locus that dropped out in Fusion analysis in grey columns, whereas the LAMP results were overlaid in green or yellow shades to show positive or negative reaction, respectively. In general, consistent positive LAMP reactions (solid green shade) correlated with full Fusion profiles (no locus dropout). In contrast, severe locus dropout was seen with consistent negative LAMP reactions (solid yellow shade). Partial Fusion dropout can be correlated with inconsistent LAMP results, where one positive and one negative reaction was seen. These were overlaid with a gradient green-yellow shade. As stated, the lowest recommended DNA for Fusion to produce a full profile was 100 pg, and the majority of the samples was below this threshold. The ability for Y-screen to detect some of these samples showcases the sensitivity of this assay. In addition, it can be concluded that Y-screen's role in identifying samples that contains male DNA can be extended to serving as a predictor for Fusion analysis success.

The dilution studies showed that Y-screen can be an effective screening tool even at dilute semen concentrations. Using three semen donors, presence of male DNA was reliably detected at dilutions up to 1:2000. At higher dilution factors, the Y-screen sensitivity was approaching its limitation. Although as Y-screen fails to detect male DNA consistently, the number of locus dropouts in Fusion analysis also begins to increase.

Given the success with efficient lysis of semen samples, and the high sensitivity and specificity of the LAMP assay for targeting human male DNA, our attention turned to analysis of mock forensic samples. This was performed in three experiments. A small sample set of mock samples (n=5) were prepared 'blind' to the analyst. Next, in addition to four swabs deposited with known ratio of male:female cells, post-coital (PC) samples with an interval (PCI) up to 72 hours were analyzed. Finally, more challenging mock samples were tested alongside samples with PCI up to 144 hours. The second sample set consisted of samples stored at -20°C, whereas the final set included samples stored at room temperature (RT) for more than 4 years. All samples were processed by Y-screen and ABAcard p30 assay, each using a quarter swab cutting.

3.B.3.d Mock sample studies for the evaluation of the Y-screen protocol

Shown in **Table 6** are the Y-screen and p30 from five blind samples. Y-screen successfully identified all four samples that contained male contributor(s) after comparing with reveal sample content. Of the five samples, 14005-1 and 14006-1 contained semen/female epithelial mixtures, which Y-screen successfully identified, but the p30 failed to identify 14006-1, which constituted of diluted semen sample. Relying on p30 assay as a confirmatory method would have disregarded sample 14006-1 as non-probative, whereas Y-screen would not. Furthermore, Y-screen detected a potential presence of male DNA in the 14001-1 sample (one positive and one negative reaction in the duplicate), which is indicative of male DNA at low quantities. The 14001-1 sample was later revealed to be male urine, suggesting that Y-screen has the sensitivity to detect dilute shed epithelial cells. The combination of identification of male DNA in a sample as well as knowledge on the origin of that sample (presence of p30), can be extremely informative. Simultaneously detecting the presence of male DNA and semen RNA (as described in the bfID section) from a single sample, by the means of DNA and RNA coextraction, therefore would be advantageous. Preliminary data suggest that the *Qiagen RNeasy* method (primary method for bfID) purifies only

Table 6 – *Comparative study of unknown samples using Y-screen and p30 assay. The table compares information deducible from Y-screen and p30 assays in comparison with the sample key. A negative result is shaded in yellow, a positive result is shaded in green, whereas a potential positive result is shaded in light green.*

Sample ID	p30 result	LAMP result	Agreement	Interpretation	Sample Revealed
14001-1	NEG	Sample may be positive for male DNA	No	This sample may have a male contributor(s) at low quantities	Male urine (neat)
14002-1	NEG	Sample does not contain male DNA	Yes	This sample does not contain male DNA nor seminal fluid	Female buccal (neat)
14003-1	NEG	Sample positive for male DNA	No	This sample contains male DNA, possibly not from seminal fluid	Male/Female buccal mixture (neat)
14005-1	POS	Sample positive for male DNA	Yes	This sample contains male DNA and is from seminal fluid	Semen/Female buccal mixture (1:20)
14006-1	NEG	Sample positive for male DNA	No	This sample contains male DNA, possibly not from seminal fluid	Diluted Semen/Female buccal mixture (1:160)

RNA, and the enzymatic lysis method (used for Y-screen) is incompatible with mRNA-based LAMP analysis. Therefore, optimization of a coextraction method would be necessary to achieve streamlined sample preparation.

The second set of Y-screen evaluation included testing of four male/female (M:F) mixtures, seven PC swabs, and one vaginal fluid swab with Y-screen compared with the p30 assay. The actual M:F ratio (as determined by qPCR) ranged over two orders of magnitude from 94 to 9929. The PC swabs had a self-reported (PCI from < 24h up to 48-60h; the time elapsed-from-swab ranged from less than 24h to a year stored at -20°C. Two quarter swab pieces were cut from each swab: one swab underwent p30 assay following the manufacturer's protocol, while the other swab was analyzed using the optimized Y-screen protocol. The results from select samples are presented in **Figure 23**, where panel A shows the colorimetric result (y-axis) against incubation time (x-axis). These semi-quantitative line graphs from four of the mixture samples and four of the PC samples show a positive reaction when the traces reach below the threshold hue value (dotted lines). The qualitative (end-point) results are logged below the line graphs

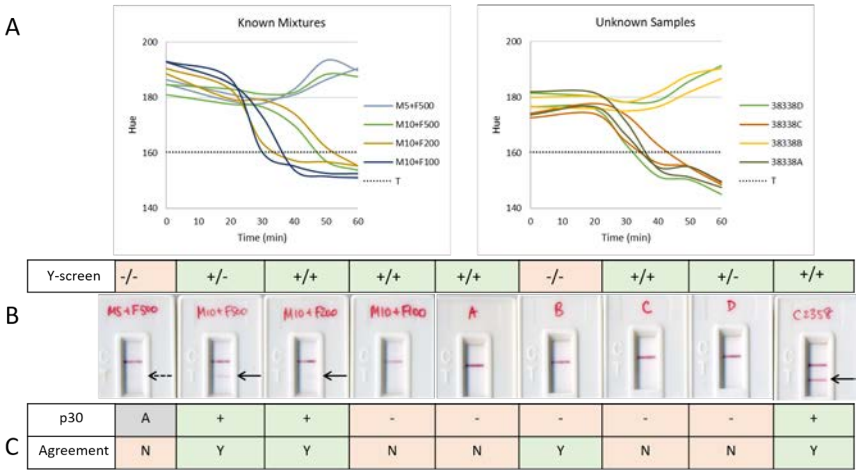

Figure 23 - *Analysis of mixture and post-coital swabs by Y-screen and p30 assay. (A) LAMP results for known M:F (left) and post-coital (right) samples. The table summarizes the duplicate reaction for each sample. (B) Scanned p30 cards with black arrow indicating a positive test line. (C) A table showing p30 results as assessed visually. The faint test line for "M5+F500" sample (dotted arrow) was labeled "A" for ambiguous.*

as either "+" for positive or "-" for a negative reaction. **Figure 23B** shows images of the p30 strips; the black arrows point to a positive reaction based on visual inspection. The dotted black arrow in the "M5+F500" sample points to a faint band at the test line, and thus was labeled as inconclusive (IC) or equivocal results. **Figure 23C** compares and logs the agreement between the Y-screen and p30 results. A complete comparison of all the tests performed on each sample is shown in **Table 7**, where the accuracy of the two screening methods was determined based on the presence of male DNA as detected by qPCR. The Y-screen method accurately detected male DNA in 10 out of 12 samples, outperforming the p30 assay which scored 8 out of 12, with no false positive results identified by either screening method. Among the mixture samples, the Y chromosomal concentration ([Y]) ranged from 0.003 to 0.022 ng/µL, compared to PC swabs which had a wider range of 0 to 0.405 ng/µL. Samples with 0.003 ng/µL were inconsistently detected by the Y-screen method, giving rise to one false negative (FN) sample ("M5+F500"). Interestingly, the same "M5+F500" sample gave a weak test line in the p30 assay, suggesting only trace male contribution on the swab. The other FN Y-screen sample, "38338E", had a [Y] of 0.405 ng/µL that was clearly within the Y-screen sensitivity, yet could not be analyzed due to an abnormal hue value (>220) (**Figure 24**). Consequently, this sample is shaded 'pink' in **Table 7** to indicate a flag was raised during analysis. A flag can also be raised during Plexor analysis, based on the disparity in Cq numbers between the test targets and the IPC. In this dataset, samples flagged by Plexor did not impact Y-screen, and vice versa, presumably due to the vast differences in the mechanism and complexities of the two assays. The indicator used in Y-screen (HNB) is tolerant to a small fluctuations in pH introduced by the sample; however, a reddish color is observed under acidic conditions[46]. The "38338E" sample was, therefore, diluted 10-fold, and this led to the expected hue response in LAMP (**Figure 24**), suggesting a possible protocol recommendation when abnormal hue is observed by the user. In this study, Y-screen correctly identified three PC samples that were not detected by the p30 assay. Two of these samples, "38338A" and "38338C, were collected at PCI 24-48h; while the third, "38338D" was collected at PCI 48-60h. The latter two were stored at -20°C for over a year after swabs were dried, demonstrating that Y-screen provides superior sensitivity for longer period PCI samples and/or aged samples.

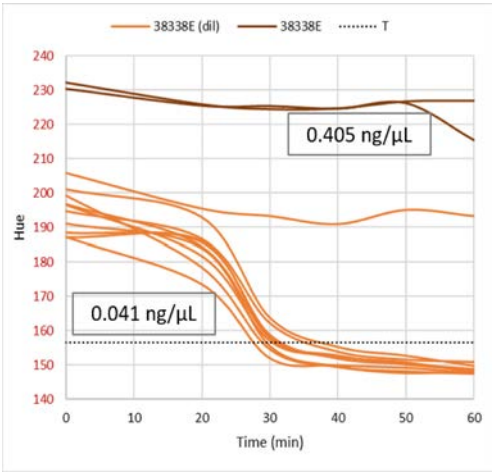

***Figure 24** - Abnormal hue in HNB LAMP reaction. Dark orange traces show abnormally high hue values for 38338E sample. Upon a 10X dilution, the light orange traces show expected hue values.*

The disagreements between Y-screen and p30 assay observed in the second study gave more insight to the capabilities (and limitations) of the protocol. However, since the samples in the study were stored at -20°C, we were concerned that these may not reflect the storage conditions for some sexual assault evidence (i.e., room temperature). Hence, as a final assessment of the Y-screen protocol, select forensically-relevant samples prepared for bfID (Error! Reference source not found.) were tested, in addition to a time interval study of PC samples collected up to 144h (total of 17 samples). These samples have been stored at RT for up to four years, and some were prepared on fabric (including dark jeans) to resemble crime scene samples. One set of samples

underwent rapid cell lysis and Y-screen LAMP, while the other set was evaluated with p30 assay. The cell lysate was quantified using Plexor HY System to determine the autosomal and Y

Table 7 - *Total sample analysis by Y-screen, p30, and qPCR. Male/Female mixture swabs and post-coital swabs were screened using the Y-screen and p30 methods, followed by a Plexor confirmatory assay. The screening results are shaded green for a positive reaction, or yellow for a negative reaction. Samples with unusual amplification were shaded pink, indicating a flagged result. The accuracy of the assays was categorized into true positive (TP), true negative (TN), false positive (FP), and false negative (FN) based on qPCR and sample preparation protocol.*

Sample ID	Sample description	p30 result	LAMP result	[Y] (ng/µL)	[Auto]	[Auto]/[Y] Ratio	Agreement	Interpretation based on combined results	Comment	Accuracy				
											TP	TN	FP	FN
M10+F100	M:F 1:10	NEG	Sample positive for male DNA	0.022	1.990	94	No	Contains male DNA, possibly not from seminal fluid	False interpretation. The sample contains semen	Y				
										PSA				
M10+F200	M:F 1:20	POS	Sample positive for male DNA	0.016	15.156	982	Yes	Contains male DNA and is from seminal fluid	One late color change in Y-screen → Bioanalyzer confirmed product	Y				
										PSA				
M10+F500	M:F 1:50	POS	Sample may be positive for male DNA	0.013	4.021	325	Yes	Contains male DNA and is from seminal fluid	One pos and one neg sample in Y-screen. May be low DNA quantity	Y				
										PSA				
M5+F500	M:F 1:100	Weak POS	Sample does not contain male DNA	0.003	31.169	9929	No	Contains trace semen fluid, but was not detectable by Y-screen	PSA verified by 2 additional observer to be a weak positive	Y				
										PSA				
38338A	PC	NEG	Sample positive for male DNA	0.003	0.846	265	No	Contains male DNA, possibly not from seminal fluid	PCI 48-60h. The seminal fluid was too diluted to be detected by PSA	Y				
										PSA				
C2358	PC	POS	Sample positive for male DNA	0.034	51.252	1507	Yes	Contains male DNA and is from seminal fluid	PCI <24h. Confirmed by both assays	Y				
										PSA				
38338B	PC	NEG	Sample does not contain male DNA	N/D	3.277	N/A	Yes	Does not contain male DNA nor seminal fluid	PCI 72h. Neither male DNA nor PSA was detectable	Y				
										PSA				
38338C	PC	NEG	Sample positive for male DNA	0.011	7.305	639	No	Contains male DNA, possibly not from seminal fluid	PCI 48h. The seminal fluid was too diluted to be detected by PSA	Y				
										PSA				
38338D	PC	NEG	Sample may be positive for male DNA	0.007	1.561	359	No	Contains male DNA, possibly not from seminal fluid	PCI 36h. The seminal fluid was too diluted to be detected by PSA	Y				
										PSA				
38338E	PC	POS	Sample flagged	0.405 (Tm outside range)	2.338	6	N/A	Contains seminal fluid, but was not detectable by Y-screen	PCI <24h. The Y-screen samples showed abnormal hue	Y				
										PSA				
38338F	PC	POS	Sample positive for male DNA	N/A	N/A	N/A	Yes	Contains male DNA and is from seminal fluid	PCI <24h. Confirmed by both assays	Y				
										PSA				
A1254	VF	NEG	Sample does not contain male DNA	N/A	N/A	N/A	Yes	This sample does not contain male DNA nor seminal fluid	Vaginal fluid was true negative for both assays	Y				
										PSA				

concentrations, and to serve as a confirmatory test. The results are summarized in, **Table 8,** where the accuracy of the two screening methods was determined based on the presence of male DNA as detected by qPCR (unless the sample origin is known, e.g., semen deposited). Similar to the previous assessment, Y-screen and p30 assay were not in complete agreement (n=7). In mock samples B001 and B009 that contained dilute semen, p30 yielded true positive (TP) results whereas Y-screen gave FN results as determined by the presence of male DNA from Plexor qPCR. The lack of amplification in Y-screen LAMP in sample B009 was uncharacteristic due to the high contributing male DNA (0.226 ng/µL), and there was no flag by Plexor to suggest inhibition. Notably, all the PC samples in this study tested negative for p30, however, five samples were deemed positive by Y-screen (B014, PC24h, PC48h, PC72h, PC96h). These samples would be disregarded as non-probative based on p30 results alone. In the PCI study where a sample was collected at 24h intervals post intercourse, and stored at RT for 2 weeks, Y-screen detected the presence of male DNA up to PCI 96h despite the low concentrations detected by Plexor.

The presence of p30 in male urine and human breast milk has been well-documented [47], thus these body fluids are often tested for potential cross reactivity [48]. Here, male urine (B019) was tested weak positive for PSA and positive for Y-screen. On the other hand, breast milk (B020) was tested negative in both assays.

Table 8 *- Total sample analysis by Y-screen, p30, and qPCR. Mock and post-coital samples were analyzed by Y-screen, p30, and Plexor. The screening results are shaded green for a positive reaction, or yellow for a negative reaction. Samples that were flagged by the Plexor internal positive control are shown with DNA concentrations in italics. The accuracy of the assays was categorized into true positive (TP), true negative (TN), false positive (FP), and false negative (FN) based on qPCR and known sample content during preparation.*

Sample ID	Sample description	Sample age	p30 result	LAMP result	[Auto] (ng/µL)	[Y]	[Auto]/[Y] Ratio	Agreement	Accuracy				
										TP	TN	FP	FN
B001	VF + Dil Se	~1 month	POS	NEG	5.421	0.008	661	No	Y				
									PSA				
B002	VF + Dil Se	~1 month	POS	POS/NEG	34.367	0.011	5880	Yes	Y				
									PSA				
B009	VF + Dil Se on jeans	~1 month	POS	NEG	33.938	0.226	150	No	Y				
									PSA				
B012	Se on filter paper	>4 years	POS	POS	23.899	16.887	1.4	Yes	Y				
									PSA				
B014	PC24h	2 months	NEG	POS/NEG	9.226	0.028	327	No	Y				
									PSA				
B015	PC48h	5 months	NEG	NEG	9.317	N.D.	N/A	Yes	Y				
									PSA				
B016	PC120h	2 months	NEG	NEG	*0.830*	*0.003*	*485*	Yes	Y				
									PSA				
B017	PC48h	2 months	NEG	NEG	16.798	N.D.	N/A	Yes	Y				
									PSA				
B019	Male urine	1 week	WEAK POS	POS/NEG	0.024	0.023	1	Yes	Y				
									PSA				
B020	Breast milk	1 week	NEG	NEG	N.T.	N.T.	N/A	Yes	Y				
									PSA				
VF	VF	1 month	NEG	NEG	*1.490*	*N.D.*	*N/A*	Yes	Y				
									PSA				
PC24h	PC24h	2 weeks	NEG	POS	4.611	0.047	99	No	Y				
									PSA				
PC48h	PC48h	2 weeks	NEG	POS	2.247	0.008	326	No	Y				
									PSA				
PC72h	PC72h	2 weeks	NEG	POS	*1.013*	*0.001*	*1594*	No	Y				
									PSA				
PC96h	PC96h	2 weeks	NEG	POS/NEG	*1.678*	*0.002*	*1201*	No	Y				
									PSA				
PC120h	PC120h	2 weeks	NEG	NEG	2.063	0.001	2566	Yes	Y				
									PSA				
PC144h	PC144h	2 weeks	NEG	NEG	*1.200*	*0.001*	*2940*	Yes	Y				
									PSA				

The second evaluation showed that Y-screen was effective at detecting male DNA at PCI 60h when samples were stored at -20°C for a year. Here, the PC sample with the longest storage time at RT (5 month) was absent in male DNA as determined by Plexor, consistent with Y-screen. PC samples B014 and B016 were stored for 2 months and had 0.028 and 0.003 ng/µL male DNA, respectively, and Y-screen was only able to detect the former. In contrast, PC samples PC48h, PC72h, and PC96h were only stored for 2 weeks, and despite having comparable Y quant as B016, Y-screen was positive. These results indicate that storage time may have a marked impact on the amplifiability of the DNA via LAMP. However, complicating this hypothesis is the semen sample deposited on filter paper stored at RT for over four years, which was detected by Y-screen (and p30) without issue.

In conclusion, we have shown the applicability and compatibility of a LAMP-based Y-screen assay involving rapid cell lysis and colorimetric detection. The lysis method is effective at yielding amplification-ready DNA from a variety of cell types in less than ten minutes, including normally resilient sperm cells. As evidenced by the PC samples tested here, nucleic acid-based Y-screen by LAMP have shown to have comparable sensitivity as commercially-available quantification kit (Plexor), and detected probative samples that otherwise would have been missed by the p30 assay. Furthermore, the Y-screen protocol can be completed in 60 min, which is roughly twice as fast as time-to-result with the p30 assay. The colorimetric indicator included in the LAMP reaction –HNB – enables quantitative detection of a positive reaction, which removes subjectivity involved in reading the test line on a p30 assay. Not unlike other tests, Y-screen does have limitations. There were incidences where a sufficient Y quant resulted in a negative reaction in LAMP (sample B009 in the final Y-screen evaluation). As discussed earlier, one sample required pH buffering prior to addition to the LAMP reaction mix to prevent abnormal HNB color change. By detecting

male DNA, a positive reaction does not reveal the specific cell type from which the sample originated. Nevertheless, we have provided proof that Y-screen is an effective tool for identifying samples containing male DNA, specifically for sexual assault evidence. Used as an alternative (or in tandem) to the p30 assay, this Y-screen approach can provide fast, accurate, and high-throughput screening of probative samples.

3.B.4 The Analytical Instrument

3.B.4.a Software

The software is critical for controlling all iLAMP functions, as well as image capture and analysis for the end-result read-out. There has been much optimization of this part of iLAMP, with the focus being primarily on two dyes: phenol red (color change from red to yellow) hydroxynaphthol blue (color change from purple to blue). Samples demonstrating a stark color change were used in developing the software, as well as those that were weaker in color change. Together these aided in defining the color spaces that would be most discriminatory between a 'positive' and 'negative' results. By and large, 'hue' from the HSB color space represents the 'shade' of a color, and is the attribute that has showed the greatest potential for robust discrimination for both dyes upon image analysis. We have optimized image analysis in the iLAMP instrument so that the closed instrument (3D-printed) (**Figure 25A**) captures an image and processes it in a spatial manner (**Figure 25C**). The iLAMP built in the last year boasts static lighting, reasonable temperature ramping, consistent temperature holds, and optimal conditions to capture images of the reactions in real-time. As such, it is a Total Analysis Instrument (**Figure 25B**).

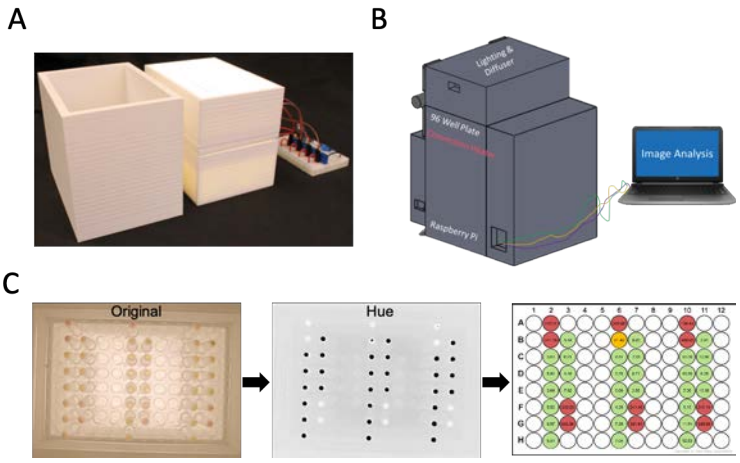

Figure 25 - *The generation-1 iLAMP Total Analysis Instrument. A: Imaging System used to optimize image analysis during LAMP amplification. B: Total Analysis Instrument design. C: Image analysis method for determining the result from a reaction. After an image is taken, the Hue values are collected and reported with a threshold for each dye that is used.*

The current generation iLAMP system runs version 5.0 iLAMP software, which has been developed with a simple graphical user interface (GUI) that allows the user to determine the total time for analysis and frequency of image capture (minimal increment 60 sec), as shown in **Figure 27**. The user specifies the file location before initiating the run, and as the run proceeds, the software automatically detects solution-occupied wells for hue analysis. In more detail, the hue value in each pixel within the well, i.e., pixel-of-interest (POI) is averaged and displayed in the GUI at specified time points. The spreadsheet file containing the data can then be exported and analyzed manually (see next section), but also has the capability for automatic data processing, including graphing, threshold determination and interpretation, to minimize manual processing.

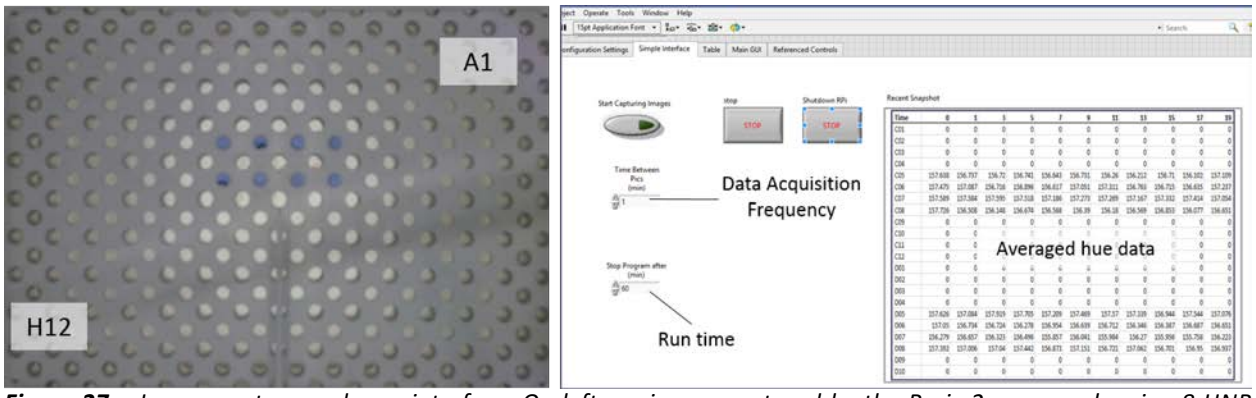

Figure 27 – *Image capture and user interface. On left, an image captured by the R pi v2 camera showing 8 HNB samples. On right, the user interface in Lab View with options and data table.*

At the end of the second year, we had honed-in on v5.0 iLAMP software that would allow for transition from the 'simple' GUI to one that allows more user options, as well as higher automated data analysis functions. More specifically, v3.0 required the user to: i) manually input temperature set-points (preheat and target temperature), ii) manually switch between preheat and running temperatures, and iii) perform data analysis off-line from the exported raw data points. In contrast, v5.0 allows the user to define basic parameters in the updated GUI (**Figure 26A**), which has embedded codes for preheat function and auto-adjusting temperature setpoint based on number of samples. During the LAMP reaction, data analysis performs 'movingaverage' (smoothing filter applied to every 3 data points) concomitant to image acquisition for real-time display of hue values. Data analysis will also calculate a dynamic threshold to determine the time-to-positive result (T_P) value for each sample, which allows a qualitative output to be presented in a 96-well array (**Figure 26B**). In proposed Phase-2 work (submitted to NIJ 4/20) will be focused on the functionality of v5.0 in integrated LAMP reactions, de-bugging of the software, and assess the intra-system and inter-system reproducibility.

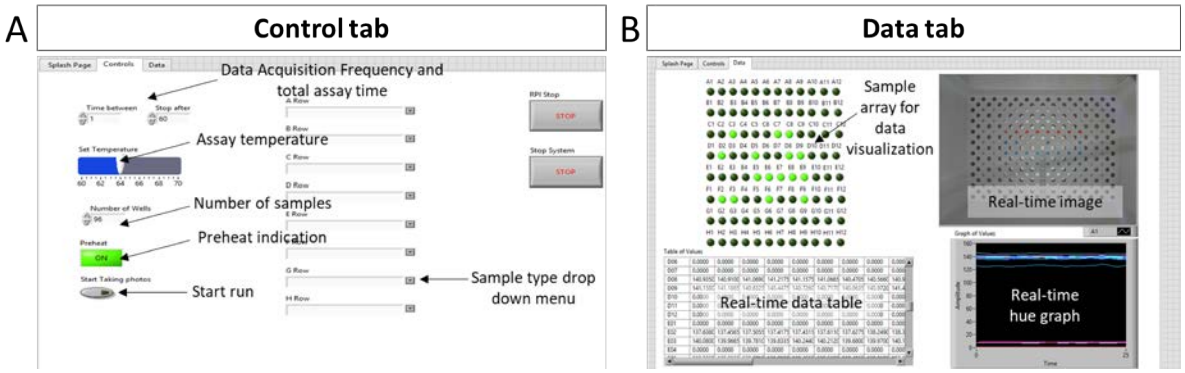

Figure 26 - *Software modifications for GUI v5.0. (A) Updated version of Control tab. (B) Data analysis information recorded during amplification.*

3.B.4.b Optimization and End-user Visuals

Currently, the integrated systems are complete with all functionality for running a LAMP assay automatically. Specifically, the systems (iLAMP-1 and iLAMP-2) will hold temperatures over 60 min, take images and perform hue analysis every minute, and identify 'positive' or 'negative' samples in real-time. When the software is first opened, the 'SplashPage' tab will be shown and the user will follow a set of prompts to start a LAMP assay (**Figure 28A)**. First, the user will be instructed to save the LAMP run under a name in the Pimount folder. Then on the 'Controls' tab,

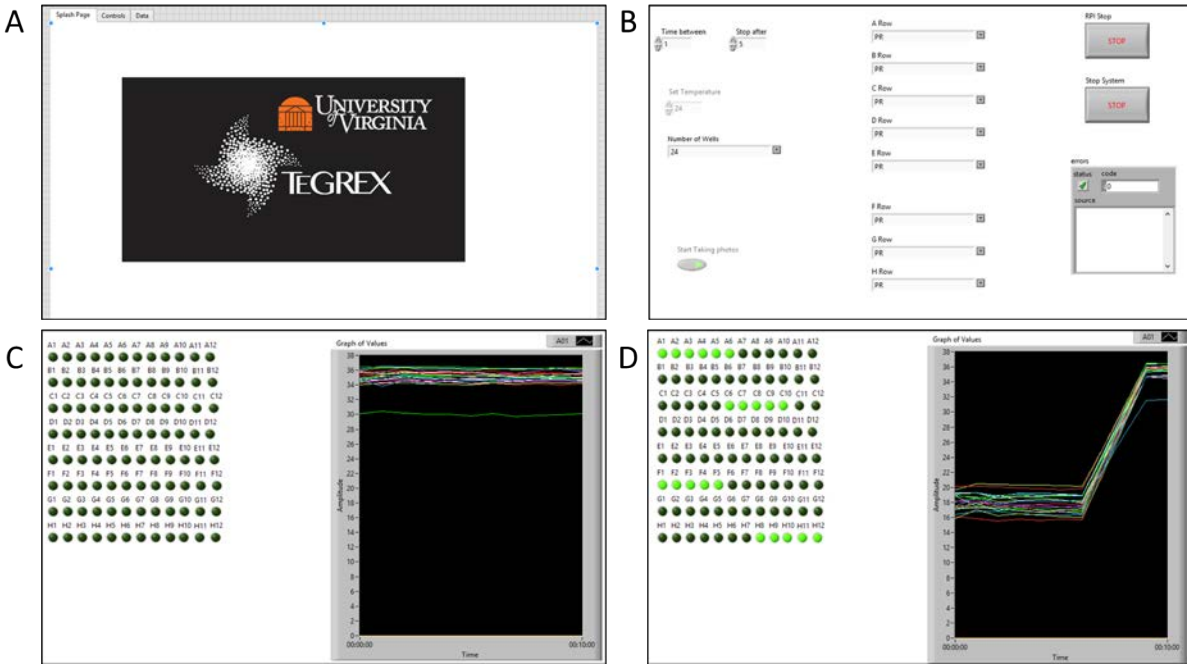

Figure 28 - *Integrated system user-visuals during a LAMP run. (A) The SplashPage tab upon opening the software. (B) The Controls tab with the parameters to input for each LAMP assay. (C-D) The Data tab showing a graph of the real-time hue analysis for all samples with indication of 'positive' or 'negative' by the lit green icons.*

the user will input the parameters for the LAMP run. The software will need to know the rate at which images should be taken (Time between = 1 image/minute), the length of the LAMP run (Stop after = 60 minutes), the desired temperature (Set Temperature = 65), the approximate number of wells based on the drop-down list, and the colorimetric dye being used in each row (PR = Phenol Red; HNB = Hydroxynaphthol blue) (**Figure 28B**). Once the parameters are set, the user inserts the reaction tubes into the integrated system and clicks the 'Start Taking photos' button. The software will then change the temperature, take image 0, and perform the first hue analysis.

After approximately 45 seconds, the hue data will begin to appear in the 'Data' tab for the analyst (**Figure 28C-D**). In the 'Data' tab, there is a graph showing real-time hue data across all sample wells and individual lights that will turn 'on' when a sample has passed the calculated threshold indicating a 'positive' result. After time points 0, 1, 2, the software will average each row and determine a threshold (**Figure 29A**). After many tests in the system, it was determined that a threshold at 3x the standard deviation was insufficient to cover all negative reactions.

A

$$Phenol\ Red\ Threshold = \left(Average\ Hue(Min\ 0,1,2)\right) + (5 * Standard\ Deviation\ (Min\ 0,1,2))$$

$$HNB\ Threshold = \left(Average\ Hue(Min\ 0,1,2)\right) - (5 * Standard\ Deviation\ (Min\ 0,1,2))$$

B

Table of Values

	0	1	2	3	4	5	6
A01	35.755	35.518	35.676	35.884	35.838	35.656	34.967
A02	35.621	35.317	35.572	35.779	35.704	35.597	36.253
A03	34.390	35.341	35.548	34.967	35.418	35.149	35.558
A04	34.625	33.846	34.606	34.086	34.832	34.593	34.462
A05	34.025	34.231	34.243	34.032	34.038	34.026	34.586
A06	34.661	34.324	34.516	34.402	34.858	34.624	34.386
A07	0	0	0	0	0	0	0
A08	0	0	0	0	0	0	0
A09	0	0	0	0	0	0	0
A10	0	0	0	0	0	0	0

Table of Values Averaged

	0.000000	1.000000	2.000000	3.000000	4.000000	5.000000	6.000000	7.000000	8.000000
A01	35.7550	35.5180	35.6760	35.6927	35.7993	35.7927	35.4870	35.0530	35.0853
A02	35.6210	35.3170	35.5720	35.5560	35.6850	35.6933	35.8513	35.5230	35.6860
A03	34.3900	35.3410	35.5480	35.2853	35.3110	35.1780	35.3750	35.0893	35.0803
A04	34.6250	33.8460	34.6060	34.1793	34.5080	34.5037	34.6290	34.6183	34.7337
A05	34.0250	34.2310	34.2430	34.1687	34.1043	34.0320	34.2167	34.1500	34.3297
A06	34.6610	34.3240	34.5160	34.4140	34.5920	34.6280	34.6227	34.4250	34.4810
A07	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
A08	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
A09	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
A10	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

Figure 29 - *Threshold calculation and exported data from the integrated system. (A) The software has the ability to calculate and use the threshold for two colorimetric dyes. Previous testing showed that a moving average of three points is sufficient for thresholding. (B) All of the hue data is exported into two csv files (Raw, Averaged) and can be seen on screen if desired.*

However, a threshold at 5x the standard deviation is sufficient coverage. At the end of the run, the software produces a compilation of files containing all 60 images, a text document logging the input parameters, a raw .csv file, and a processed .csv with averaging applied. An example of the data in the two *.csv files are shown in **Figure 29B**, which allows for the user to compare the files and do a manual analysis, if requested. Having access to these files allows the users to tabulate the data as they wish and troubleshoot the assay if needed.

3.B.4.c Hardware

We built four iLAMP instruments (**Figure 30A**) and, in the final year was in the process of building a fifth. The Total Analysis Instrument consists of a 3D printed ABS enclosure, a forced convection heating system, an imaging system, direct front lighting, diffused backlighting, and a user interface with an automated image analysis program. The iLAMP Instrument is simple to operate: the user loads samples into the sample plate, opens the LAMP virtual instrument in the National Instruments LabVIEW software on the laptop, specifies a name for the test, defines a picture interval, enters an end time for the test, and presses the start button. The iLAMP will then run

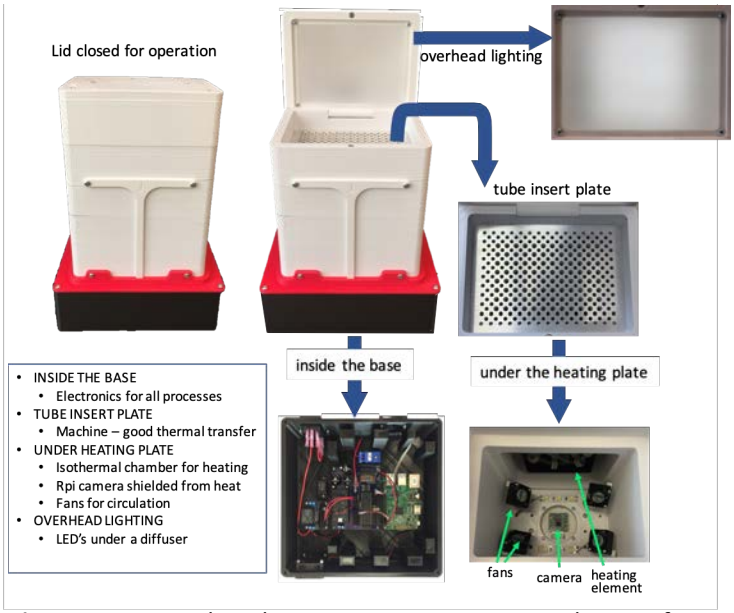

Figure 30 - *A: Total Analysis Instrument. B: Heating element, fans, direct front lighting, and camera. C: CNC machined sample plate. D: Diffused backlighting.*

This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

until the test is complete and save all of the data from the test in a single folder with the user specified name.

The iLAMP system is very simple to operate: the user loads samples into the sample plate, opens the iLAMP protocol in the laptop-driven LabVIEW software, specifies a name for the test, defines a picture interval, enters an end-time for the test, and presses the 'start' button. The iLAMP system will then run until the assay is complete, and saves the data from the assay in a single folder with the user-specified name.

3.B.4.d Temperature Stability Testing

After the final systems were built with forced air convection heat/cooling, and the software deemed compatible for initial testing, we evaluated stability to hold at the desired temperature over 60 mins. In addition, we evaluated the ramp rate to reach target temperature. Both are critical for reducing the chance of NSA. Twenty-four low-profile PCR tubes were filled with 12.5 µL water, with eight tubes probed with a type-T thermocouple to be monitored simultaneously. During testing, we found that the ramp rate to reach the necessary in-tube temperature was 0.2°C/sec, which required ~180 sec to ramp from ambient to reaction temperatures. This observation was tightly coupled with NSA in the iLAMP system that, otherwise, was not observed on the thermocycler. We, therefore, hypothesized that the slow temperature ramp rate can contribute to mis-matched annealing to non-target DNA strands, therefore, leading to NSA. In latter part of year-1, we reported a ramp rate of 1°C/sec by modifying the design of the sample plate to allow better metal-to-sample contact surface area. In the final version of the prototype, we further improved the ramp rate by preheating at a setpoint higher than the LAMP reaction temperature for 45 minutes. By doing so, we managed to reach a ramp rate of ~3°C/sec, which is faster than many conventional thermocyclers (**Figure 31**). Day-to-day temperature variability was also studied, and found that there is around 0.5°C fluctuation within a well, an amount that we deem acceptable for LAMP reactions. There is also temperature variation spatially on the 96-well plate, although this was observed both on the integrated system as well as the conventional

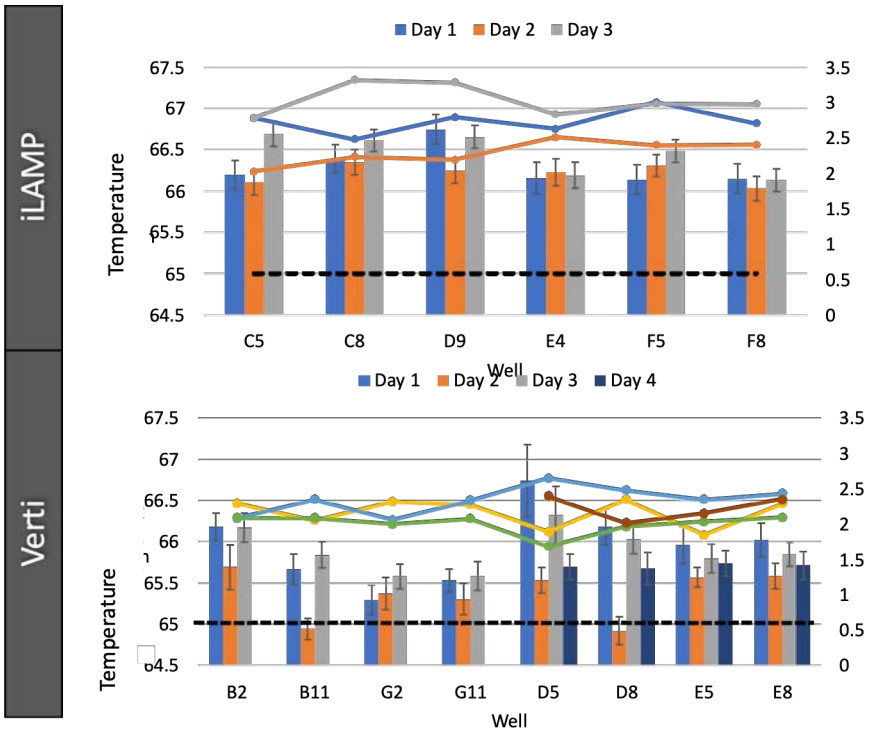

***Figure 31** - Comparing average temperatures across wells in the Veriti thermal cycler and Integrated system. The ramp rates are similar in both systems and the average temperature is one degree higher in the integrated system.*

thermocycler. In addition, the variation is not associated with a particular well, suggesting the variation came from the experimental setup rather than the hardware itself. As shown in **Figure 31**, we optimized the system to hold an average of ~66 °C across wells, this is again to prevent any NSA prevalent at lower temperatures (**Figure 31**). Temperature variability over a long term (weeks to months) or susceptibility to environmental temperature was not tested.

Strategies that improved ramp rates: 1) increased metal-to-sample contact surface area (v2.51 in **Figure 32**), and 2) an elevated preheat step to account for heat lost during sample loading. The combinatorial effect was positive on temperature ramp rate; a five-fold increase from the previous version (v2.4 in **Figure 32**). However, the improved metal-to-sample contact surface area comes with an inherent narrowing of the tube opening, thus, posing a challenge for image analysis. Hence, the software for imaging needed refining to assess whether these strategies successfully removed the NSA associated with the iLAMP systems.

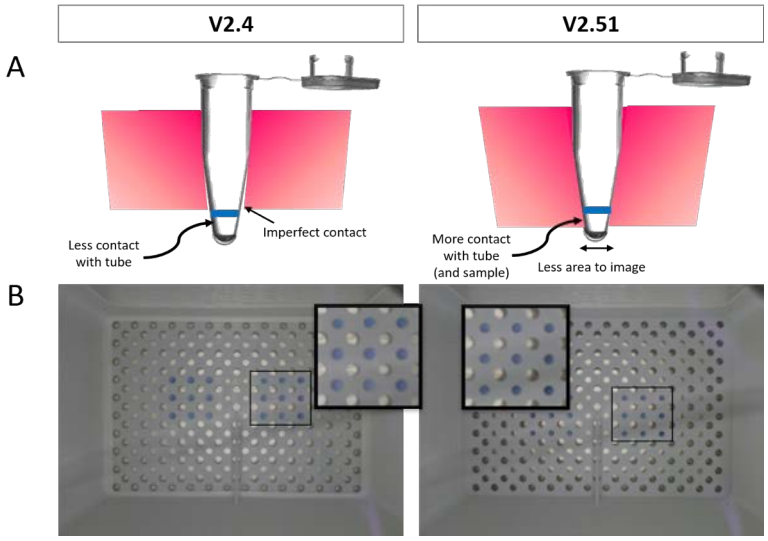

Figure 32 - *Hardware modification. (A) Increased metal-to-sample contact surface area. (B) The increased contact surface area gives a smaller diameter of the reaction for image analysis.*

3B.4.e Data Generated with the iLamp Instrument

Preliminary testing on the iLAMP system has been done with two dye chemistries (described in **Figure 1**) and targeted multiple body fluids. **Figure 33** shows target-specific amplification of

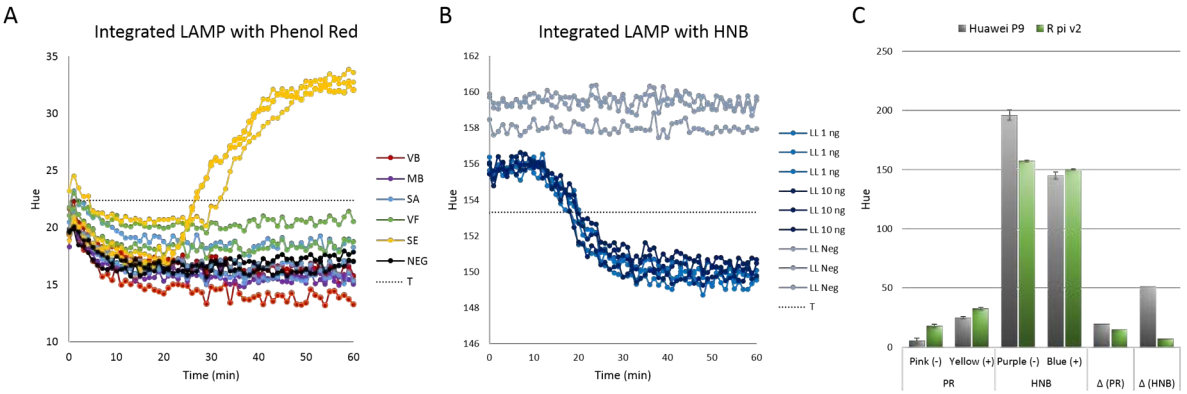

Figure 33 - *Integrated LAMP runs. Left, LAMP with phenol red where the hue values increase if samples become positive. Middle, LAMP with HNB where hue values decrease if samples become positive. Right, the averaged hue values for positive and negative reactions for both indicators and the differential in hue values for each setup.*

semen at 63 °C (**Figure 33A**) and male DNA at 65 °C (**Figure 33B**), suggesting that temperature homeostasis at these set points can be achieved in the iLAMP system for the duration of the assays. Success in automated well detection and averaging of hue values negated the manual imaging and analysis steps, however, graphing and threshold determination were carried out manually. The thresholds in **Figure 33** were defined as three standard deviations from the average hue values at 10 or 0 min for phenol red and HNB, respectively. Phenol red can exhibit small hue fluctuations with ambient temperature even though the reagents appear pink to the naked eye, thus the threshold was set using the hue values at 10 min (once the reagents reached the set point). One notable difference with HNB-based colorimetric analysis on the iLAMP system using the Raspberry pi (R pi) v2 camera is that the hue differential between a negative and positive reaction is lower compared to the camera on Huawei P9 phone (a much more expensive camera) (**Figure 33C**). Specifically, the differential between positive and negative reactions on the R pi was ~7 in comparison to 50 on Huawei P9. The differential for phenol red was also lower on the R pi, but not as extensive. This discrepancy may be due to a lower blue pixel digital number in the IMX219 CMOS in the R pi v2.[49] Post imaging processing, e.g., electronic tinting, has been tested to increase the HNB differential, although more testing is required to determine whether tinting is necessary to distinguish between violet (negative) and blue (positive) colors. In order to extract the time-to-positive (Tp) values from the amplification plots, smoothing can be carried out using a 'moving average' of 4 data points; as shown in **Figure 34**, this provides more clarity in defining the time when a sample adopts a color that is 'positive'. The T_P value can be viewed as analogous to the 'Cq value' in real time PCR, which provides indication as to the quantity of the starting template in the sample.

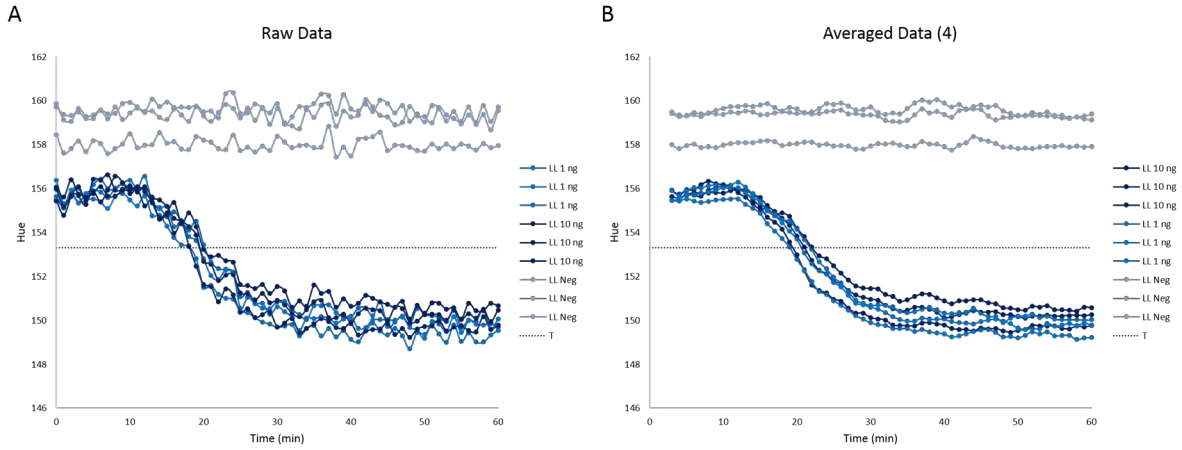

Figure 34 - *Data processing. (A) Raw data without smoothing. (B) Moving average applied to every 4 data points to allow smoothing without sacrificing resolution or accuracy of data.*

3.B.5 Concluding Remarks

We have met the majority of the proposed specific aims, having: 1) developed a colorimetric bfID assay that was successful for venous blood, saliva and semen, 2) developed a colorimetric Y-screen assay, and 3) built prototype instruments capable of executing the LAMP assay with colorimetric detection. Based on feedback from two forensic labs, we dispensed of menstrual blood as a target. Vaginal fluid remains a

significant fluid for identification, and would be valuable added to the panel, but it has been challenging and will require additional research to define targets that provide specificity.

Overall, this LAMP body fluid identification panel, despite omission of VF, has significant potential for providing contextual information by accurately and rapidly identifying blood, saliva, and semen in unknown samples. Three highly discriminatory mRNA markers were used to design LAMP assays that were amplified efficiently at the same temperature, with a specificity that was validated as reproducible for identification of each target body fluid among multiple users. We believe the elegance of this approach is that thermocycling is circumvented, in combination with the use of simple dyes for colorimetric read-out with simple cameras including a smart phone as the detector. We challenged the LAMP method with dry stains on denim, as well as with azospermatic samples – both performed remarkably well for successful detection. The blind study that was carried out provided a preliminary validation with respect to the efficacy of the method when performed by individuals who are not highly trained as scientists. Relative to existing methods, this provides an accelerated sample-to-answer method for mRNA with high specificity and sensitivity (single copies of RNA) and, with further development, could provide unparalleled bandwidth (five fluids) for body fluid ID.

In terms of DNA-based Y-screening with emphasis on sexual assault samples we provide a novel alternative to current methods. A rapid (<10 min) enzymatic sperm cell lysis efficiently releases nucleic acids in a simple closed-tube lysis that is amenable to scale-up into 96- or 384-well format that could easily be interfaced with robotic processing. The enzymatic lysis protocol was recently validated in New York's Office of Chief Medical Examiner for improving sexual assault sample triaging by selecting the 'optimal' sample for STR profiling [3]. Here, the compatibility of the crude semen lysate with two LAMP chemistries (HNB and phenol red dyes) was demonstrated, as well as standard Taqman quantification and commercially-available forensic DNA quantification kit (*Plexor*). Using the appropriate primers, the Y-LAMP assay is highly specific, as indicated by no false positive results observed with non-human samples or with up to 30 ng of human female DNA. The advantages of this nucleic acid-based Y-screen method, is that it does not require fluorophore-tagged primers, thus, negating the requirement of complex and expensive detection systems. Consequently, this presents the possibility of a more cost-effective methodology when processing a large number of samples, i.e., the sexual assault sample backlog.

Finally, we present an iLAMP system, including the software for operation, that allows for the automated execution of the LAMP assay on a 96 well/tube format. While this is a laboratory-based prototype that used approaches that would expedite development and testing (like a 3D-printed shell and other parts), it was built by an engineer with a view to commercialization in mind. Hence, the gap between prototype and an industrial instrument that could commercialized is small.

3.B.5 Future Efforts

Future efforts have three facets. The first facet is to trial two of the iLAMP systems, along with the 3-body fluid panel assay, in two forensic labs. This would have been completed by now, but the COVID crisis has stalled activities. The second and third facets involve 'R&D' that is described in a proposal we submitted to the NIJ this past spring. The 'R' involves the completion of the research needed to clear the hurdles we encountered with VF, and obtain the necessary VF specificity to yield a powerful 4-body fluid assay panel. In addition, we are considering extrapolation to other body fluids, such as urine and sweat. The 'D' will involve creating an industrial-quality instrument that can easily be commercialized. This is possible as a result of the engineering on the prototype created with the current round of funding, which was designed to have subsystems and built with parts that could translated to scaled-up manufacturing.

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