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Development of a Loop-Mediated Isothermal Amplification (LAMP) Assay for Detection of
miRNAs

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Abstract

Body fluid identification serves a vital role in contextualizing biological evidence received in forensic laboratories. While several fluids can be presumptively detected using immunological or catalytic tests, these tests are susceptible to false results and have limited sensitivity. True confirmatory testing is still somewhat limited in forensic laboratories. With the advancement of DNA typing technologies, laboratories can develop DNA profiles from trace amounts of sample, increasing the demand for sensitive and accurate body fluid detection. This project proposes using loop-mediated isothermal amplification (LAMP) as a method to rapidly detect microRNAs (miRNAs) that are differentially expressed in various body fluids with high specificity and without the need for specialized equipment. LAMP detection of miRNAs specific to semen and blood were evaluated, testing multiple LAMP template and primer methodologies for their specificity and sensitivity. An assay design utilizing a template DNA with a complementary region for its respective target miRNA was moderately successful. With this design, the target miRNA initiated strand displacement synthesis, prompting exponential amplification. A positive control containing 10^6 - 10^{11} copies of synthetic miRNA could be detected in 45 minutes in both real-time qPCR and gel systems with little to no amplification of a negative control containing the respective template DNA. This window was extremely narrow since amplification of the negative control typically followed within mere minutes. A few methods were evaluated to see if this non-specific amplification could be alleviated by reducing primer and template DNA concentrations, and by adding reagents such as diethylformamide (DEF) and dimethylsulfoxide (DMSO) to improve specificity. The addition of DMSO and DEF slightly improved specificity, but further validation is necessary. With additional optimization, this assay could demonstrate proof of concept for the development of a rapid on-site body fluid identification assay.

Keywords

Loop-mediated isothermal amplification (LAMP), forensic science, miRNA, body fluid identification, blood, semen

Introduction

As the first step in the processing of biological evidence, serological testing and body fluid identification is of great interest in the forensic community. The identification of body fluids in forensic samples can aid in crime scene reconstruction and provide crucial evidence in an investigation. In particular, the detection of blood or semen can be invaluable evidence in violent crimes or sexual assault cases. Certain catalytic or immunological tests can presumptively identify the presence of such body fluids, but confirmatory testing is still somewhat limited.

Serological Methods for Body Fluid Identification

Current methods for presumptive detection of blood and semen include the phenolphthalein test or luminol test for blood and the acid phosphatase (AP) test or prostate-specific antigen (PSA) lateral flow assay for seminal fluid. These assays are widely practiced in a typical forensic workflow, but they are limited in their specificity, often consume a large amount of sample, and have a high incidence of false positive results (1). Since the phenolphthalein test for blood relies on the peroxidase-like activity of hemoglobin to catalyze a colorimetric reaction, this test can also react positively to certain plant peroxidases and common household cleaners containing oxidizing agents (1). Furthermore, even the PSA lateral flow assay is vulnerable to false results. One study found that the PSA test yielded 44% false positives and 20% false negatives among 132 sexual assault samples compared to polymerase chain reaction (PCR) methods targeting DNA markers on the Y chromosome (2).

Molecular Body Fluid Identification

Molecular methods for body fluid identification offer a more sensitive approach that could increase confidence in proper classification. These identification methods rely on the detection of tissue-specific markers such as mRNA or miRNA expression, DNA methylation levels, and the identification of microbial species (3).

Some of the variation between tissue types can arise from variations in the epigenome, or modifications in the form of DNA methylation, histone modification, or chromatin structuring. Unlike other modifications, DNA methylation can be detected following DNA extraction and is the most compatible epigenetic marker with standard forensic protocols (3). Specific and reliable methylation markers have been discovered for semen containing spermatozoa (4,5) and markers for blood, saliva, menstrual blood, and vaginal fluids have been identified with varying levels of specificity (4,5). In general, epigenomic methods for body fluid categorization are sensitive and accurate but limited when the recovered DNA is degraded or single stranded, both of which are common in forensic analysis due to the condition of the evidence, or the extraction method used.

Body Fluid Identification via mRNA Expression

Cell differentiation during embryonic development is driven primarily by differences in the types and sequences of genes that are transcribed to messenger RNAs (mRNA). Differences in gene expression originate from interactions between transcription factors, enhancer or silencer regions in the DNA, and variations in chromatin density that ultimately impact which genes are transcribed into mRNA (and later translated into protein). As such, each cell type expresses a subset of coding genes that make up the full transcriptome. This lends itself to the use of mRNAs as a target for the identification of tissues (3); however, mRNA methods have not achieved

widespread acceptance in the forensic community due to the perceived challenges of working with mRNA. Body fluid identification via mRNA sequencing has been implemented into forensic casework in Europe, New Zealand, Asia, and Australia (6–8); however, a validated method has not been approved for casework in the United States.

Body Fluid Identification via microRNA Expression

As an alternative to mRNA-based assays, microRNA targets are a recent area of interest in body fluid identification. MicroRNAs (miRNA) are a class of small, non-coding RNAs that regulate post-transcriptional gene expression and other cellular processes. These small RNAs typically work in the cytoplasm to regulate translation through mRNA silencing or cleavage primarily through interactions with the 3' untranslated region of the targeted mRNA (9). The genes encoding miRNAs are located throughout the genome and are frequently expressed co-transcriptionally, thus generating tissue-specific expression patterns (3,10). Tissue and body fluid specific expression of miRNAs has been utilized to identify potential biomarkers for cancer and other pathologies (11), and this has also been applied towards body fluid identification in forensics (12–19).

The characterization of forensic tissue-specific miRNAs has been evaluated using microarray and reverse transcription quantitative PCR (RT- qPCR) analysis; however, a lack of a consensus on tissue-specific markers could have resulted from differences in platforms, detection chemistries, and normalization to different endogenous reference miRNAs between forensic studies (12–19). Wang et al. (20) was the first group to describe a high-throughput sequencing (HTS) application to forensic body fluid identification when they performed high-throughput small RNA sequencing of blood and saliva. Expanding on this research, HTS analyses performed by Seashols-Williams et al. identified miRNAs that are differentially expressed in blood, seminal

fluid, saliva, feces, menstrual secretions, vaginal fluid, perspiration, and urine (21). Additionally, the miRNAs let-7g and let-7i were indicated as potential endogenous reference genes with consistent expression across multiple body fluids (21). A 10-fold cross-validated quadratic discriminant analysis (QDA) model has been utilized by this laboratory to evaluate classification accuracies, reporting a miRNA panel that can classify blood, menstrual blood, feces, urine, saliva, semen, and vaginal fluid with an average accuracy of 93% (22). Accuracy of body fluid predictions were higher in single source fluids collected from different donors or the same donor over various time points, but classification accuracy was reduced when mixed body fluid samples were analyzed (22).

Further analysis of let-7g and let-7i in both RNA extracts and DNA extracts indicated that miRNAs can be detected in DNA extracts, albeit at lower concentrations (23). This follows previous findings of Omelia et al. and van der Meer et al. (24,25), who described miRNA body fluid identification methods capable of distinguishing miRNAs specific to blood and saliva from DNA extracts. Historically, miRNA analysis has required a separate RNA extraction step, which consumes additional sample. Since every effort should be made to conserve the starting sample in a forensic setting, removing the intermediate RNA extraction step could allow miRNA analysis to be implemented into forensic casework with minimal disruption to the DNA analysis workflow.

With the increased research into miRNAs as a biomarker for forensic body fluid identification, the need for sensitive and specific detection methods has come to light. While the short sequence lengths contribute to miRNA stability, this also makes them a difficult analytical target with limited potential for multiplexing. Additional challenges include large variability in per-cell copy number and high sequence similarity between families of miRNAs (26).

Traditional methods such as northern blotting, and microarray analysis are still in practice but can be time consuming and lack the sensitivity needed to be an efficient detection method. Quantitative PCR (qPCR), long considered the gold standard of amplification-based detection, is a sensitive assay but requires specialized equipment and precise thermal cycling. As an alternative to PCR, loop-mediated isothermal amplification (LAMP) has been reported in literature as a sensitive and rapid amplification technique for the detection of nucleic acids, including miRNAs (26–31).

Loop-Mediated Isothermal Amplification

LAMP is an isothermal reaction that can be used to selectively amplify DNAs and RNAs with high sensitivity. This method typically uses four to six primers to hybridize to multiple sequences on the target template simultaneously, leading to exponential amplification through concatemerization (26). In LAMP-based approaches to detecting miRNAs, the miRNA initiates the reaction through auto-cycling strand displacement DNA synthesis through the recognition of distinct primer binding sites (32). At the end of this process, multiple double stem-loop structures with various stem lengths are generated, and these in turn can initiate subsequent LAMP (28). Because each stem-loop product contains multiple sites for the initiation of synthesis, this process is significantly faster than the product doubling seen in each cycle of PCR. The entirety of the reaction can be performed at one temperature, which eliminates the need for the same type of thermal cycling parameters as PCR. This enables the use of more portable and less expensive equipment, which could be utilized in forensics to achieve precise body fluid identification at the crime scene.

High amplicon yields contribute to the overall versatility of LAMP detection methods. The accumulation of large amounts of DNA and amplification by-products allows for detection

of LAMP products by turbidity measurements, agarose gel electrophoresis, or real-time detection utilizing intercalating or colorimetric dyes. Detection of LAMP products using gel electrophoresis is often used as a preliminary method to indicate whether the assay is working as expected. LAMP end products consist of stem-loop DNAs with inverted repeats of the target and structures with a cauliflower-like appearance containing multiple loops. When detected on a gel, the products will create a ladder-like pattern indicative of amplicons of various sizes (32). As a real-time detection method, the presence of a white precipitate caused by the accumulation of magnesium pyrophosphate, a byproduct of LAMP, can be monitored using a turbidimeter or using the naked eye (33,34). This visual method of detecting LAMP products is unique to LAMP reaction chemistry since PCR does not generate the same type or magnitude of byproducts. LAMP byproducts can be similarly detected using pH indicating dyes such as phenol red, or with hydroxynaphthol blue, which changes color as free magnesium in the reaction is consumed as amplification progresses (34). Fluorescent intercalating dyes such as SYBR Green can be used to directly detect double stranded DNA products, and are commonly utilized in combination with a real-time PCR system (28,31,34).

LAMP is a widely utilized method for isothermal amplification, appearing in over 3,700 scientific publications and 8 clinical trials (35). In recent years, LAMP has emerged in the biomedical community as a molecular diagnostic tool for diseases such as pulmonary tuberculosis and SARS-CoV-2 (35,36). LAMP has been demonstrated to rapidly detect SARS-CoV-2 RNA at low levels in 30 minutes or less, prompting the development of commercialized kits and tests such as New England Biolabs' SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit and Lucira Health's at-home COVID-19 test kits. The recent interest in LAMP as a diagnostic

tool for COVID-19 has brought the method, originally developed in 2000 (32), to the forefront of the scientific community.

LAMP Body Fluid Identification

To this point, research on LAMP as a method for identification of forensically relevant body fluids has been primarily focused on mRNA targets. Real time reverse transcription-LAMP has been investigated as an approach to detecting body fluid specific mRNA targets (37,38). The Landers laboratory at University of Virginia has developed LAMP protocols to allow for rapid detection of body fluid specific mRNA targets and human male DNA through the colorimetric detection of LAMP products (39–42). The mRNA targets for venous blood, semen, and saliva were primarily detected using phenol red, a color changing pH indicator that can indicate whether amplification has occurred based on the pH of the reaction mixture (40). Phenol red changes from red to yellow as DNA polymerase incorporates free dNTPs into the growing DNA strand and releases hydrogen ions as a byproduct, altering the pH of the reaction (43). This color change is observable to the naked eye and has also been quantified using a specialized colorimetric image analysis method that uses a 3D-printed imaging system and a smartphone camera to measure changes in the hue of LAMP reactions containing phenol red (39,40). Likewise, human male DNA can be detected using LAMP to screen for the Y-linked Amelogenin region of DNA (42). In addition to phenol red, hydroxynaphthol blue (HNB) was used as an indicator for amplification, signaling that amplification has occurred through a color change from violet to blue as the amount of free magnesium in the reaction decreases.

The sensitivity and speed of LAMP together with the stability and high copy number of miRNA targets show potential for the development of a rapid and sensitive body fluid identification assay. LAMP miRNA detection offers superior sensitivity and accuracy and could

be performed in parallel to the typical DNA profiling workflow following DNA extraction. Furthermore, detection-based molecular assays such as LAMP could be mobilized in a way that allows for body fluid identification in settings outside of a laboratory (for example, at a crime scene). Recent advances in the optimization of LAMP detection of miRNAs have simplified the workflow and improved overall sensitivity of the assay. Tian et al. (28) developed a method of combining rolling circle amplification (RCA) and LAMP to detect miRNA within a limit of 10 aM. In this method, the presence of target miRNA facilitates the hybridization of a padlock probe and miRNA to form circular single-stranded DNA. The circular DNA will undergo RCA when a series of primers and DNA polymerase are added. The resulting RCA product contains long tandem sequences that can form stem-loop structures, as well as initiate reverse extension reaction upon hybridization with a stem-loop primer. This design (depicted in Figure 1) allows each target miRNA to generate several stem-loop structures with various stem lengths, all of which can independently initiate LAMP (28). The RCA reaction and LAMP reaction are performed in a single step without any intermediate transfer steps, making it a simple method for miRNA detection. RCA-LAMP proposes an effective method for detecting short RNA molecules, such as miRNA; however, the major limitation of this assay is low amplification accuracy as LAMP DNA template can be amplified through non-specific DNA synthesis from the padlock probe and primers (28,44). In the original RCA-LAMP study, this was demonstrated by the fact that some amount of DNA amplification was detected in the miRNA-negative samples (28).

Other methods for miRNA detection with LAMP include a one-step LAMP method described first by Li et al. (31), depicted in Figure 4. Within this method, a template DNA is synthesized containing the sequences of the primers and a region that is complementary to the

miRNA target of interest. Initially, the forward inner primer (FIP) hybridizes to the F2c region on the template DNA and Bst DNA polymerase extends along the template in the presence of dNTPs. Subsequently, the miRNA will hybridize to the complementary region upstream of F2c in the template and initiate strand displacement DNA synthesis (31). As strands are displaced, complementary regions can fold over on themselves, allowing the formation of double stem loop structures that can further interact with forward and backward inner primers (FIP/BIP), prompting the formation of more step-loop structures of various stem lengths. Overall, this method was found to be capable of detecting miRNA down to 1 amol, and could differentiate miRNAs from the let-7 family with high specificity (31).

The objective of this project was to design and optimize a LAMP assay that could detect miRNAs specific to blood and semen by expanding upon existing protocols within the literature for LAMP of miRNA targets. To date, LAMP has not been utilized for miRNA analysis in a forensic setting, yet it is an ideal assay for the development of a rapid on-site screening tool. By demonstrating that miRNAs can be amplified with high sensitivity and specificity, this assay could potentially be optimized in a way that would allow for molecular body fluid identification in the field.

To evaluate the utility of a LAMP assay for body fluid identification, two methods for detection of miRNA utilizing different template and primer methodologies were evaluated: RCA-LAMP and a one-step LAMP protocol. The specificity and sensitivity of each method was examined through temperature and time course experiments targeting miRNA sequences in synthetic RNA oligos and RNA extracts from multiple body fluids. Gel electrophoresis was used as a preliminary detection method to visualize amplification products, followed by real-time fluorescent detection using SYBR Green.

Materials and Methods

Sample Selection and Preparation

The miRNA targets analyzed include those previously found to be differentially expressed in blood (miR-144-3p) and semen (miR-891a), in addition to the endogenous reference miRNA let-7i (19–21). Investigated samples included synthetic RNA oligonucleotides as well as RNA extracts from the blood and semen from various donors. Synthetic RNA oligos were designed to mimic the mature miRNA sequences of miR-891a, miR-144-3p, and let-7i-5p (Table 1), including a modification for 5' phosphorylation. All three synthetic oligos were synthesized and HPLC purified by Integrated DNA Technologies (Coralville, Iowa).

Donated body fluid samples were collected in accordance with VCU human subjects IRB protocols (HM20002931). Blood samples were collected on sterile cotton swabs, dried, and stored in swab boxes at room temperature. Semen was collected in sterile collection cups supplied to the donor and returned on ice within 24 hours. Semen was aliquoted onto sterile cotton swabs in volumes of 50 μ L, which were dried and stored at room temperature until RNA extraction could be performed. RNA extraction of collected body fluid swabs were performed using the AllPrep DNA/RNA Mini Co-Extraction Kit (Qiagen, Hilden, Germany) and RNA extracts were stored at -80°C until ready for use.

Primer Design- RCA-LAMP

LAMP primer sets were designed based on the published sequences by Tian et al. (28). The stem-loop primer (SLP), forward inner primer (FIP), and backward inner primer (BIP) were synthesized to be identical to the published sequences. The padlock probe hybridization sequences were modified to be complementary to the following miRNAs: hsa-let-7i-5p, miR-

891a, and miR-144-3p (Table 1). The padlock probes for these microRNAs were designed to have complementary regions on the 5' and 3' ends of the probes. The 5' end of the padlock probe consisted of the last half (10-11 bases) of the reverse complement of the mature miRNA sequence. The 3' end of the probe contained the reverse complement of the first half of the mature miRNA. The sequences of the RCA-LAMP primers and probes are listed in Table 2. All nucleic acids were synthesized and purified via standard desalting by Integrated DNA Technologies.

RCA-LAMP miRNA Assay

The RCA-LAMP protocol used within this project was a variation of that used by Tian et al. (28) using reagents from the WarmStart LAMP Kit (New England Biolabs, Ipswich, MA). The first step of RCA-LAMP involved pre-ligation of the padlock probe by combining 1X T4 RNA Ligase 2 buffer and 1 U T4 RNA ligase (New England Biolabs); 2 nM padlock probe; 4 U ribonuclease inhibitor (Thermo Fisher Scientific, Waltham, MA); and 2 μ L extract or the equivalent copy number for synthetic oligos. These components were mixed and incubated at 39°C for 30 minutes in the ProFlex™ PCR System (Thermo Fisher Scientific) to complete the ligation reaction.

Following ligation, 2 μ L of the ligation reaction products were added to the RCA-LAMP mixture containing 100 nM stem-loop primer (SLP), 0.8 μ M forward inner primer (FIP), 0.8 μ M backward inner primer (BIP), and 12.5 μ L WarmStart LAMP 2X Master Mix (New England Biolabs) and brought up to a total volume of 25 μ L with DEPC treated water (Quality Biological Inc, Gaithersburg, MD). The reaction mixture was placed in the ProFlex Thermal Cycler to incubate the RCA-LAMP reaction at a constant temperature of 67°C for up to 60 minutes, followed by termination at 85°C for 5 minutes, and a 4°C hold.

Following the persistence of non-specific amplification of the padlock probe during parameter optimization, a new LAMP master mix was evaluated consisting of 1X ThermoPol Buffer, 8 mM MgSO₄, 1.4 mM dNTPs, 8U Bst DNA Polymerase Large Fragment, 0.8 μM FIP, 0.8 μM BIP, and 100 nM SLP. All master mix reagents, excluding primers, were obtained from New England Biolabs. All reactions were brought up to 25 μL with DEPC treated water (Quality Biological Inc). The ligation reaction components and incubation parameters remained unchanged from previous validation work.

Primer Design- One-Step LAMP

The primers for the one-step LAMP assay approach utilized sequences published by Li et al. (31). The sequences for the forward inner primer (FIP), backward inner primer (BIP) and backward outer primer (B3) remained unchanged from those in the literature. The template DNA was altered to contain the reverse complement sequence to the miRNA target (let-7i-5p, miR-891a, miR-144-3p) on the 3' end of the template DNA sequence (Table 3). The primers and template DNA were synthesized and purified with PAGE by Integrated DNA Technologies.

One-Step LAMP miRNA Assay

Following parameters established by Li et al. (31), LAMP reactions were set up in 0.2 mL strip tubes containing 50 pM template DNA, 0.6 μM FIP, 0.6 μM BIP, 50 pM B3 primer, 1X ThermoPol Reaction Buffer, 0.2 mM dNTPs, 4 U Bst DNA Polymerase Large Fragment, 2 mM MgSO₄ (New England Biolabs), 1M betaine (Sigma Aldrich, St. Louis, MO), and 8U ribonuclease inhibitor (Thermo Fisher Scientific). All reactions were made up to a total of 10 μL in DEPC treated water (Quality Biological Inc). Positive control reactions contained 1 μL of synthetic miRNA (10¹¹ copies/μL). As a negative control, a blank and a NTC were performed

with every sample set. The blank contained all reaction components except synthetic oligo or extract, and the NTC contained all master mix components except template DNA and miRNA. All RNA extracts tested were added to the reaction mixture at a volume of 2 μ L.

Prior to LAMP amplification, a preheating step was performed to denature any double stranded template DNA (31). This was performed by first combining all reaction components except Bst DNA polymerase to a total volume of 8.5 μ L (0.8 μ L 10X ThermoPol Reaction Buffer, 0.3 μ L 20 μ M FIP, 0.3 μ L 20 μ M BIP, 0.5 μ L 1 nM B3, 0.2 μ L 100mM MgSO₄, 2 μ L 5 M Betaine, 0.4 μ L 20 U/ μ L RNase inhibitor, 0.2 μ L 10 mM dNTPs, appropriate volume of synthetic oligo or RNA extract, and water to reach a total volume of 8.5 μ L). This reaction mix was incubated on the ProFlex™ PCR System for 95°C for 5 minutes, followed by 10 minutes at 50°C. The reactions were allowed to cool to room temperature naturally (on the bench) for about 10 minutes. The cooled reaction mixture was then placed on ice and mixed with 1.5 μ L of a mixture containing 0.2 μ L 10X ThermoPol Reaction Buffer, 0.5 μ L 8000 U/ mL Bst DNA Polymerase Large Fragment, and 0.8 μ L DEPC treated water, for a total reaction volume of 10 μ L. LAMP reactions were incubated again on the ProFlex™ PCR System at 55°C for the duration of the reaction.

When real-time detection with SYBR Green was desired, the preheating step was omitted and 1X LAMP Fluorescent Dye (New England Biolabs) was added to the reaction mixture. To alleviate issues with early amplification in the real-time amplification plots, the enzyme was changed to 4 U Bst 2.0 WarmStart DNA Polymerase and its corresponding 1X Isothermal Amplification Buffer (New England Biolabs). All reaction components remained unchanged outside of this substitution.

Gel Electrophoresis

Gel electrophoresis was used as a preliminary method to detect the presence of amplicons in completed LAMP reactions. A 2% agarose gel (Gold Biotechnology, St. Louis, MO) stained with 1X SYBR Safe (Thermo Fisher Scientific) was prepared and run in 1X TBE buffer (VWR International, Radnor, PA). Samples were prepared in volumes of 12 μL to load into the gel, with 2 μL 6X Blue/Orange Loading Dye (Promega, Madison, WI), 7 μL 1X TBE, and 3 μL of the LAMP reaction products. Additionally, 5 μL of 100 bp PLUSTM DNA Ladder (Gold Biotechnology) was added to the gel. Once prepared, the agarose gel was run at 120 V for approximately 70 minutes, and then imaged on the UVP BioDoc-It Imaging System (Analytik Jena, Jena, Germany) at 302 nm.

Detection with the Quant Studio 6 Real Time PCR System

LAMP reactions were set up as previously described in a 96 well plate and sealed with a MicroAmp Optical Adhesive Film (Applied Biosystems, Waltham, MA). The plate was centrifuged for approximately one minute to remove any bubbles, and then placed in the QuantStudioTM 6 Flex Real-Time PCR System (Applied Biosystems). The run method created on the instrument included a PCR stage that cycled through 10 seconds at 60°C, followed by 20 seconds at 60°C (capture step) for a total of 135 cycles. Following the PCR stage, the instrument ramped up to 80°C for 5 minutes to terminate the reaction during the hold stage. The maximum ramp speed of 2.8°C/second was selected for both stages of the LAMP protocol. Lastly, melt curve analysis was performed using the standard melt curve settings (95°C for 15 seconds, 60°C for 1 minute, slowly ramp to 95°C at a rate of 0.05°C/second while capturing data). Since a reporter dye was not used, analysis of amplification plots was performed by viewing reporter signal (Rn) vs cycle.

Results and Discussion

RCA-LAMP Parameter Optimization

Preliminary tests using the RCA-LAMP assay design focused on detecting a synthetic let-7i sequence (identical to the mature miRNA sequence listed in Table 1) without amplification of the padlock probe. In theory, the RCA-LAMP design relies on the ligation of miRNA to a linear padlock probe, forming circular single-stranded DNA that can undergo rolling circle amplification (Figure 1). RCA will generate a long stretch of DNA that can interact with LAMP primers to generate stem loop structures that are detectable by a variety of methods. In the absence of the miRNA complementary to the ends of the padlock probe, RCA should not be initiated, and this process should be restricted.

Synthetic RNA was selected as a positive control for all LAMP experimentation since it should have less background signal and trace genomic DNA, allowing for simpler optimization of the assay and an appropriate measure of the assay's efficiency when run alongside RNA extracts from body fluids. Negative controls included a ligation no template control (NTC) that contained the padlock probe without miRNA and a LAMP NTC that contained only the master mix and primers. These controls were selected to investigate the amplification specificity; a successful assay would demonstrate amplification of the positive control that can be distinguished from negative controls containing the padlock probe. The LAMP NTC should not amplify, as this would suggest primer dimerization or reagent contamination.

Unfortunately, initial tests conducting LAMP for 1 hour at 67°C and 70°C showed amplification of positive samples as well as the ligation NTC (Figure 2). The LAMP NTC did not have visible amplification products, suggesting that primer dimerization is likely not

responsible for non-specific amplification. Some amount of amplification of the padlock probe may be expected due to the presence of regions complementary to the LAMP primers within the probe (Tian et al. (28); Table 2). The results obtained in Figure 2, however, suggest that amplification of positive samples and the padlock probe could not be distinguished. Due to the decreased resolution of the amplicon bands observed in the gel when LAMP was run at 70°C, 67°C was selected as the standard temperature for RCA-LAMP going forward.

To investigate if the non-specific amplification was time dependent, a time course was performed by performing RCA-LAMP at 67°C for 15, 30, 45, and 60 minutes using the same sample set containing a positive control, a ligation NTC, and a LAMP NTC (Figure 3). This experiment indicated that amplicons were not detectable in a gel system after 15 minutes of LAMP, but any amplification of the positive samples containing 10^8 copies/ μ L of synthetic let-7i was accompanied by non-specific amplification of the ligation NTC containing the padlock probe. Further testing with an expanded control sample set revealed that when the padlock probe was input directly into the LAMP reaction without first going through ligation with miRNA or extract, the probe amplified in a comparable manner to positive samples and the Ligation NTC (data not shown). It was hypothesized that if the padlock probe was not given sufficient opportunity to ligate to any miRNA present in the reaction that this could have contributed to non-specific amplification; however, heating and slowly cooling the probe and miRNA to promote annealing prior to the addition of ligase did nothing to alleviate the issue. Furthermore, this pattern was observed with both let-7i and miR-891a, indicating that this assay design was flawed for both targets.

One-Step LAMP Optimization and Time Course

One-step LAMP is a method initially established by Li et al. (31) that follows a simpler approach to the detection of miRNA through a linear single-stranded DNA template DNA containing a complementary region for the miRNA target (Table 3). The miRNA acts as the forward outer primer as it interacts with the template and displaces the strand synthesized beginning with the forward inner primer (Figure 4). As such, the presence of miRNA should initiate the exponential amplification of LAMP products.

To evaluate the effectiveness of this method for let-7i-5p, miR-891a, and miR-144-3p, a time course was performed using the temperature and reaction concentrations established by Li et al. Reactions were incubated simultaneously at 55°C for 30 minutes, 45 minutes, or 60 minutes on three separate blocks of the Proflex PCR System. Following incubation, reactions were terminated at 80°C for five minutes, then kept on a 4°C hold until all reactions were completed. The initial runs were performed targeting synthetic let-7i, and this was repeated for a total of three replicates (Figure 5A). This time course revealed that selective amplification of the positive control with little to no amplification of the blank was possible around the 45-minute mark. It should be noted that the NTC did not amplify at any point during the time course. The process was repeated for three replicates targeting miR891a, with similar results (Figure 5B). When miR144-3p was targeted, amplification of the blank was detectable at the 45-minute mark (Figure 5C). This target was only run for a total of one replicate since it was determined that at this point that gel electrophoresis was not a sensitive enough detection method to be able to isolate the exact point at which the blank began to amplify. Consistent with other studies, this time course suggested that positive reactions and template-containing blanks could be

differentiated by the time it takes them to amplify, marking a significant turning point for the project.

Detection of microRNAs in RNA Extracts

Prior to switching to a real-time detection system, the one-step LAMP method was applied to detecting miRNA in extracts from blood, semen, and saliva to evaluate whether detection could be extended beyond the synthetic oligos examined to this point. While let-7i seemed to show stable expression across all fluids (Figure 6B), this was overshadowed by the weak amplification of the blank in the same gel. Without being able to eliminate the possibility that the detection of let-7i in these samples is due to non-specific amplification of the template DNA, the results were determined to be unreliable until the non-specific amplification problem could be resolved. While the blank did not have visible products when miR-891a (a semen marker) was targeted (Figure 6A), these results show expression levels that are inconsistent with previous research since RNA extracts from blood and saliva also showed significant amplification. Without a functioning endogenous reference miRNA, these results should also be considered with some skepticism.

Real-time SYBR Green Detection

To determine the exact point at which amplification occurs in both positive and negative LAMP reactions, real-time detection with SYBR Green (New England Biolabs LAMP Fluorescent Dye) was utilized. Initial experiments (not shown) using the same parameters as preliminary gel electrophoresis experiments yielded amplification plots with high levels of fluorescence, but no distinct curves. The high fluorescence suggested the presence of double-stranded DNA products, but the absence of curves indicated that amplification could have been

occurring prior to loading the plate onto the PCR instrument. As such, Bst 2.0 WarmStart DNA polymerase was used for all runs moving forward. Following this switch, amplification plots displayed distinct curves that could be interpreted.

Figure 7 shows the results of LAMP detection of let-7i in a sample set containing a positive control (10^6 copies of synthetic let-7i), blank, NTC, and RNA extracts from blood, saliva, and semen. Similar to the results observed in gels, the NTCs did not amplify, but the blank amplified around 5 cycles or 3 minutes after the positive control (Figure 7A). This would explain the inconsistent results in the time course gels if significant amplification of the blank can be detected within minutes of the positive control. Let-7i was detectable in the RNA extracts (Figure 7B); however, without eliminating non-specific amplification, it is difficult to determine whether these data are reliable. In order to get accurate detection in extracts, there should be better separation of the positive control and the blank, similar to data observed by Li et al. (31).

Attempts to mitigate the non-specific amplification of the blank included decreasing concentrations of the template DNA and B3 primer (Figure 8). It was believed that decreasing the template and B3 primer would decrease the opportunities for LAMP primers to interact with the template in the absence of the miRNA target; however, decreasing the amount of template and B3 primer from 50 pM down to 25 pM, 12.5 pM, and 6.25 pM did little to increase the separation between positive control and blank.

Other attempts to alleviate non-specific amplification included decreasing the concentration of betaine in LAMP reactions from 1 M to 0.5 M (Figure 9A) followed by removing it altogether (Figure 9B). Betaine has been used in LAMP reactions previously to reduce secondary structure formation and base stacking, improving strand separation and amplification efficiency (28,31,45). In theory, betaine therefore increases specificity of

amplification. To test whether non-specific amplification of the template DNA could be the result of strand breathing, the amount of betaine was decreased to see if this would have a beneficial effect on distinguishing the non-specific amplification from positive samples. On the contrary, decreasing betaine from 1 M to 0.5 M did little to the amplification specificity (Figure 9A), and removing betaine altogether restricted all amplification of positive and negative controls (Figure 9B).

DMSO was added to LAMP reactions at concentrations of 1% (Figure 10A) and 5% (Figure 10B). DMSO has been reported to increase specificity of LAMP and PCR by limiting inter and intrastrand re-annealing (46–48). The effectiveness of DMSO on LAMP efficiency does seem to vary, since Gao et al. noted adding 5% DMSO only postponed the turning point of non-specific LAMP by 3.11 minutes (44), whereas Wang et al. noted that 7.5% DMSO could inhibit non-specific amplification of LAMP (47). Here, adding 5% DMSO restricted all amplification (Figure 10B). This is consistent with the claim made by Wang et al. that excess DMSO can inhibit the activity of Bst 2.0 WarmStart DNA polymerase. Conversely, 1% DMSO had a slightly favorable effect, causing the blank to amplify approximately 5 cycles (or 3 minutes) after the positive control (Figure 10A), where the normal parameters yielded a blank and positive control that amplified around the same time. The addition of DMSO yielded a very slight improvement, and further validation would need to be performed to ensure consistency.

To investigate the effects of adding a formamide derivative, diethylformamide (DEF) was added to LAMP reactions at concentrations of 1% to 3%. Formamide, or specifically, dimethylformamide, has been demonstrated to increase specificity in PCR by binding to the grooves of DNA and destabilizing the double helix structure, thereby lowering the melting temperature and reducing opportunities for non-specific priming (49). Diethylformamide was

selected for LAMP based on findings by Scott et al. (50) that suggested that DEF minimized non-specific amplification in LAMP caused by non-specific priming. A concentration series of 1%, 2%, and 3% DEF was selected based on these findings. Overall, 1% DEF was the most promising concentration for improving separation between the positive control and the blank (Figure 11). When 2% DEF was added, the blank unexpectedly amplified before any other samples (not shown), and 3% DEF caused the positive control to amplify after the blank. The addition of 1% DEF did not affect the timing of the blank amplification compared to samples prepared without DEF; however, the positive control amplified slightly earlier. This change was not significant but could indicate the potential for DEF to be used in combination with another additive to remedy non-specific amplification.

Conclusions

Applying LAMP towards the detection of body fluid specific miRNAs can prove useful in the development of a rapid screening tool to identify body fluids both inside and outside of the laboratory. While LAMP is widely regarded as a promising tool for nucleic acid detection, false positive results are still frequently observed due to the considerable number of primers required and the assay's high amplification efficiency (46). An additional challenge was designing a LAMP assay that could detect a target that is only 20 to 22 nucleotides long, requiring the use of additional probes or template DNAs complementary to the miRNA target. Since the probe contains regions that can interact with the primers regardless of whether the miRNA target is present, this presents an additional challenge in attempting to minimize non-specific amplification. Notably, the incidence of false positives, limited suitability for small targets, and complex primer design are the most frequently reported disadvantages of LAMP in the literature (35).

Overall, the RCA-LAMP assay evaluated within this project was unsuccessful due to its complicated design and persistence of non-specific amplification of the padlock probe across multiple reaction conditions. It is possible that the amplification of the probe resulted from failure to ligate and form circular DNA. As a remedy, one area of future research could involve the addition of exonuclease I to digest any remaining single stranded linear DNA.

The one-step LAMP assay was slightly more successful, demonstrating that a positive control containing 10^6 - 10^{11} copies of synthetic miRNA could be detected in 45 minutes at 55°C with little to no amplification of the blank containing the respective template DNA when end-point analysis using gel electrophoresis was used. This was confirmed in a real-time detection system, although the window in which amplification of positive samples occurred without amplification of the blank was extremely narrow (approximately 3 minutes) and not suitable for detection of miRNAs in extracts at this point. Some attempts were made towards increasing the separation between the positive control and blank, including increasing reaction temperature to 60°C and using a WarmStart Bst DNA polymerase; decreasing the concentrations of template DNA, B3 primer, and betaine; and adding DMSO or DEF. Of these attempts, the addition of 1% DMSO or 1% DEF had the most promising results, but additional validation is needed.

Continued optimization of this assay is necessary before it can be used as a screening tool for miRNAs. Future projects should aim to maximize the separation between reactions containing miRNA and negative controls. This could include incorporating a combination of DMSO and DEF in LAMP reactions, since preliminary results indicate that DMSO might delay amplification of blank samples, while DEF slightly increases amplification of positive samples. Should this fail to improve conditions, it might be necessary to redesign primer and template sequences to achieve better specificity. Additionally, real time detection using miR-891a should

be performed. Optimization of let-7i detection is necessary as the endogenous reference miRNA; however, LAMP products visualized on gels indicate that the let-7i blank amplified periodically at 45 minutes between different replicates, whereas the miR-891a blank was consistently undetectable at this time point. Following optimization of all positive and negative controls, future studies should examine the sensitivity and specificity of the LAMP assay through evaluation of varying amounts of extracts from different body fluids. Lastly, fluids from multiple donors should be tested to ensure that target miRNA can be reliably detected across donors.

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Figures and Tables

miRNA	miRbase Accession ID	Mature miRNA sequence (5'-3')
hsa-let-7i-5p	MIMAT0000415	UGAGGUAGUAGUUUGUGCUGUU
miR-891a	MIMAT0004902	UGCAACGAACCUGAGCCACUGA
miR-144-3p	MIMAT0000436	UACAGUAUAGAUGAUGUACU

Table 1: Mature miRNA sequence and accession ID for target miRNAs.

miR-891a was used as a semen marker, miR-144-3p was used as a blood marker, and let-7i-5p was used as an endogenous reference miRNA.

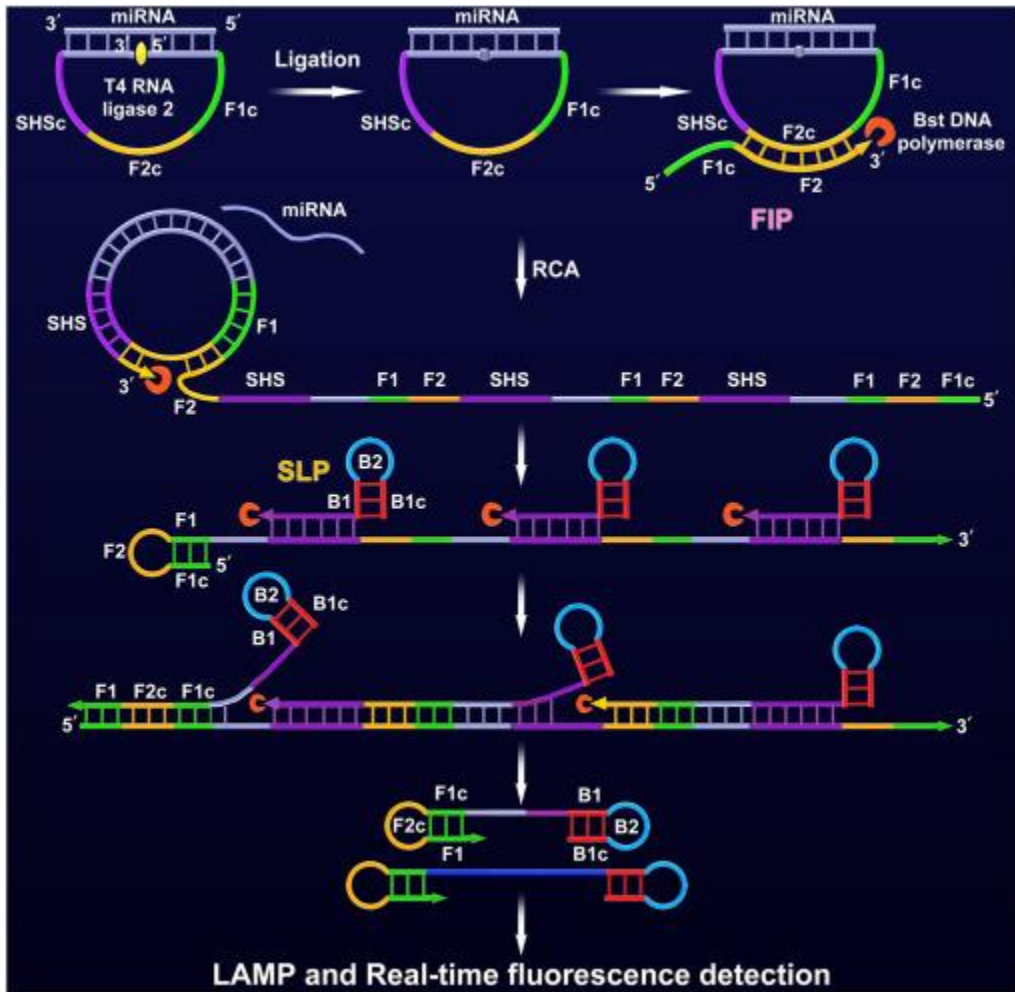


Figure 1: Depiction of RCA-LAMP method for miRNA detection.

The sequences for the miRNA-specific padlock probes, FIP primer, BIP primer, and SLP primer are listed in Table 2. Figure reprinted from Tian et al. (28).

Name	Sequence
hsa-let-7i-5p Padlock Probe	5'-phosphate- <u>CTACTACCTCA</u> TTTT CGACACGACACGA TTTT GGA ACTCTGCTCGACGGATT AAAATAAATACAGTCTGCCACAACC TTTTAACAGCACArArA -3'
miR-891a Padlock Probe	5'-phosphate- <u>GGTTCGTTGCA</u> TTTT CGACACGACACGA TTTT GGA ACTCTGCTCGACGGATT AAAATAAATACAGTCTGCCACAACC TTTTTCAGTGGCTrCrA -3'
miR-144-3p Padlock Probe	5'-phosphate- <u>CTATACTGTA</u> TTTT CGACACGACACGA TTTT GGA ACTCTGCTCGACGGATT A AAATAAATACAGTCTGCCACAACC TTTTAGTACATCrArU -3'
SLP	5'- ATCGTCGTGACTG TTTT CCCTAACCC TAACCCTAACCCTTTT CAGTCACGA CGATTTTTT AATACAGTCTGCCACAACC-3'
FIP	5'- CGACACGACACGA AAAAAATCCGTCGAGCAGAGTTCC-3'
BIP	5'- ATCGTCGTGACTG TTTT CCCTAACCC TAACCCTAACCC-3'

Key: ■ F1c ■ F2/F2c ■ SHSc ■ B1/B1c ■ B2

Table 2: Primer and padlock probe design for RCA-LAMP detection of let-7i-5p, miR-891a, and miR-144-3p.

Complementary sequences are color coded (see Figure 1 for a schematic of primer hybridization sites). In the padlock probe, the bold underlined sequences indicate hybridization regions for the designated miRNA target. The letter 'r' indicates ribonucleotides.

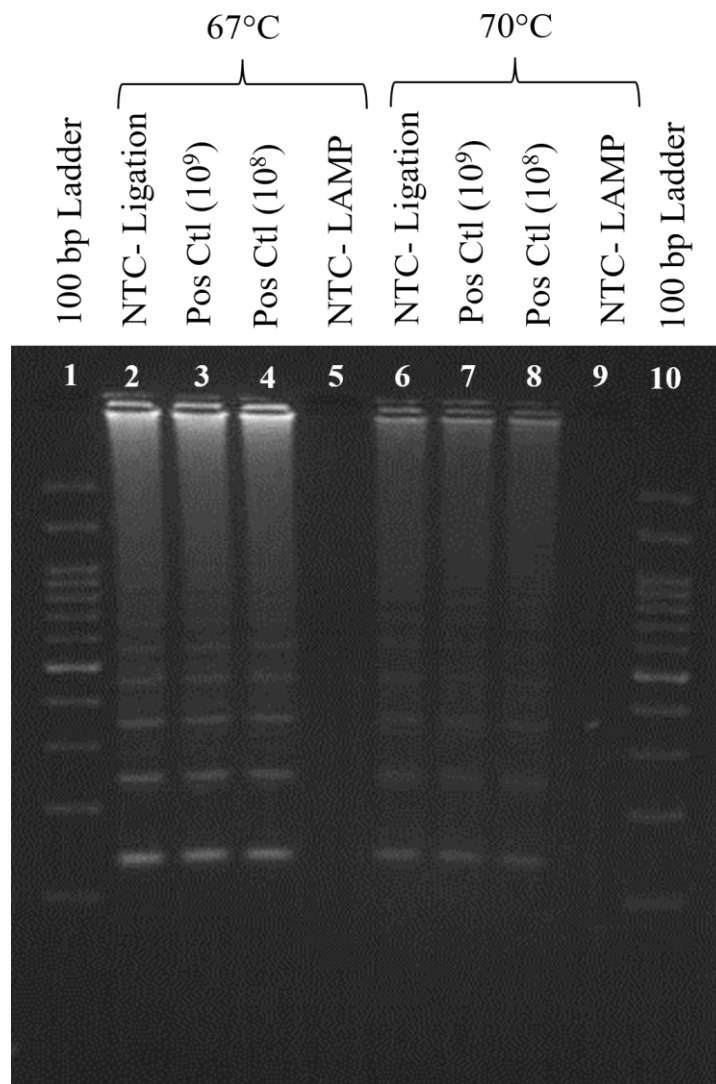


Figure 2: Reaction temperature comparison of RCA-LAMP detection of let-7i.

All reactions were incubated for 1 hour at either 67°C or 70°C. LAMP no template control (NTC-LAMP) samples (Lanes 5 and 9) were prepared by adding water to the LAMP reaction instead of ligation products, and therefore these samples did not contain padlock probe or miRNA. Ligation no template control (NTC-Ligation) samples (Lanes 2 and 6) contain padlock probe and were prepared by adding water to the ligation reaction in place of miRNA. Positive control samples (Lanes 3, 4, 7, 8) contained all reaction components and either 10⁸ or 10⁹ copies/μL of synthetic miRNA.

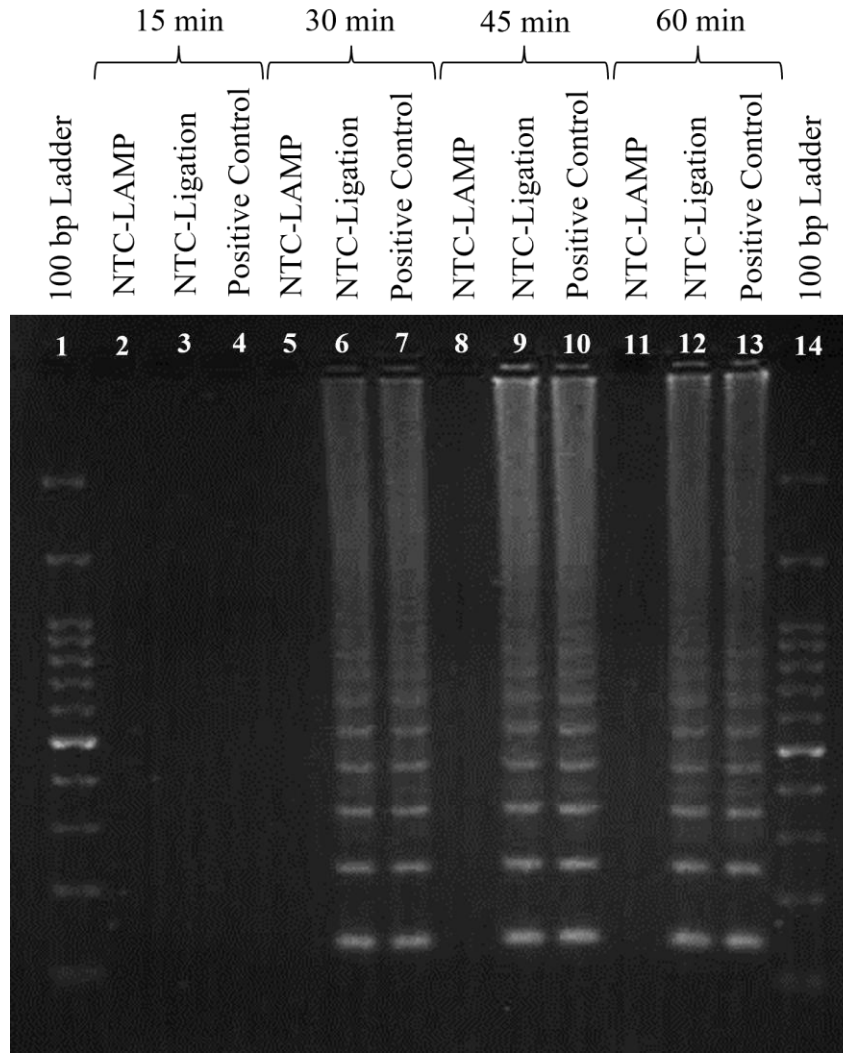


Figure 3 : RCA-LAMP time course detection of let-7i performed for 15-60 minutes at 67°C.

LAMP no template control (NTC-LAMP) samples (Lanes 2, 5, 8, 11) were prepared by adding water to the LAMP reaction instead of ligation products, and therefore these samples did not contain padlock probe or miRNA. Ligation no template control (NTC-Ligation) samples (Lanes 3, 6, 9, 12) contain padlock probe and were prepared by adding water to the ligation reaction in place of miRNA. Positive control samples (Lanes 4, 7, 10, 13) contained all reaction components and 10^8 copies/ μ L of synthetic miRNA.

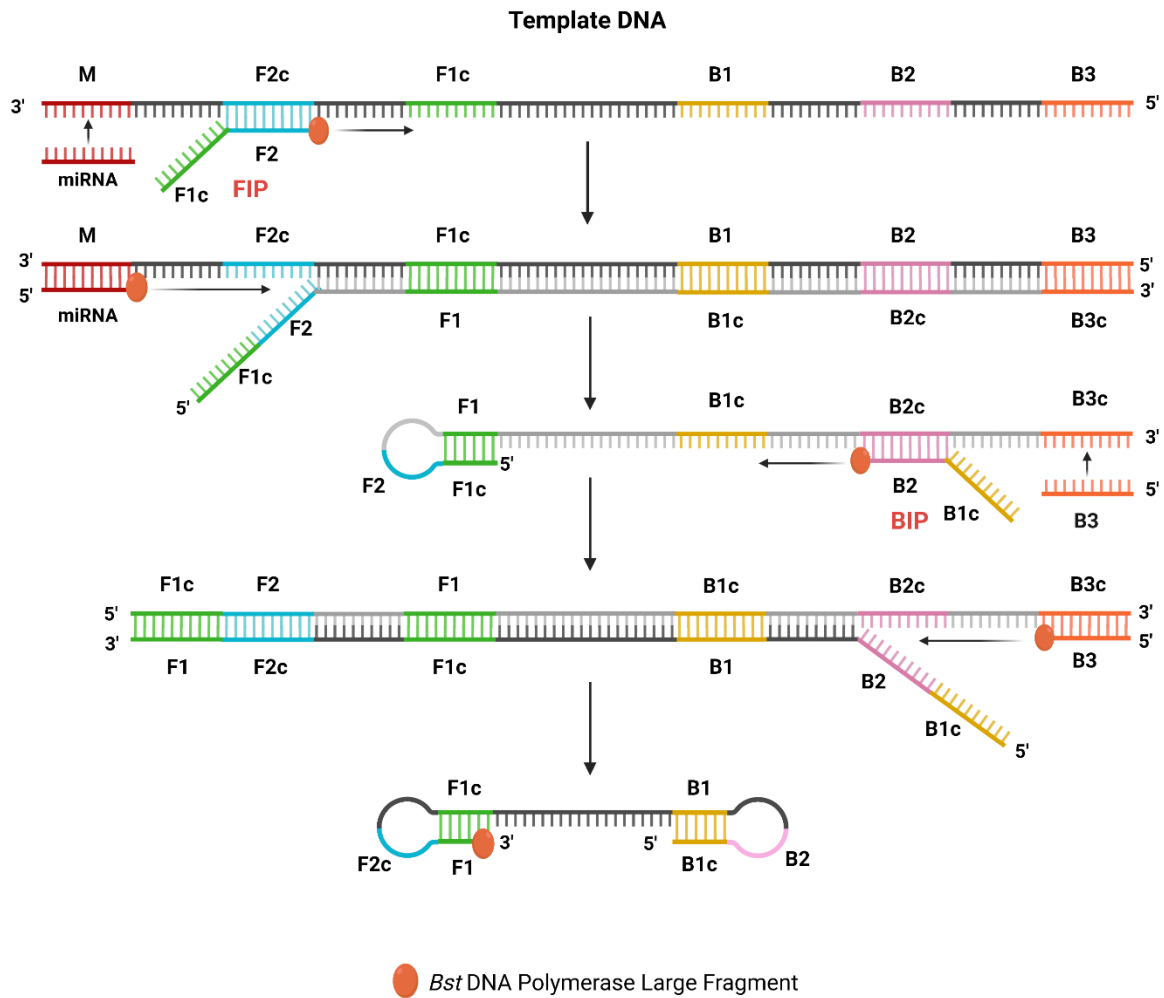


Figure 4: Schematic illustration of the one-step LAMP reaction initiated by the target miRNA.

The sequences of the miRNA-specific DNA templates, FIP primer, BIP primer, and B3 primer are listed in Table 2. Figure adapted from Li et al. (31) and generated in BioRender.

Name	Sequence
FIP Primer	5'- ACAACGTCGTGACTGGGAAAACCCTTTTGTGCGGGCCTCTTCGCTAT TAC-3'
BIP Primer	5'- CGACTCTAGAGGATCCCCGGGTACTTTTGTGTGTTGGAATTGTGAGC GGAT-3'
B3 Primer	5'-ACTTTATGCTTCCGGCTCGTA-3'
let-7i-5p Template DNA	5'- CTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACA ATTTCACACAGGAAACGTACCCGGGGATCCTCTAGAGTCGACCTGCA GGCATGCAAGCTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACC CAACTTAATCGGTAATAGCGAAGAGGCCCGCACAAACAGCACAAACT <u>ACTACCTCA</u> -3'
miR-891a Template DNA	5'- CTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACA ATTTCACACAGGAAACGTACCCGGGGATCCTCTAGAGTCGACCTGCA GGCATGCAAGCTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACC CAACTTAATCGGTAATAGCGAAGAGGCCCGCACTCAGTGGCTCAGG <u>TTCGTTGCA</u> -3'
miR-144- 3p Template DNA	5'- CTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACA ATTTCACACAGGAAACGTACCCGGGGATCCTCTAGAGTCGACCTGCA GGCATGCAAGCTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACC CAACTTAATCGGTAATAGCGAAGAGGCCCGCACAGTACATCATCTA <u>TACTGTA</u> -3'

Key: F1c F2/F2c B1/B1c B3 B2

Table 3: Primer and template DNA design for one-step LAMP detection of let-7i-5p, miR-891a, and miR-144-3p.

Complementary sequences are color coded (see Figure 4 for a schematic of primer hybridization sites). Bold underlined regions in the template DNA indicate regions complementary to their respective miRNA targets.

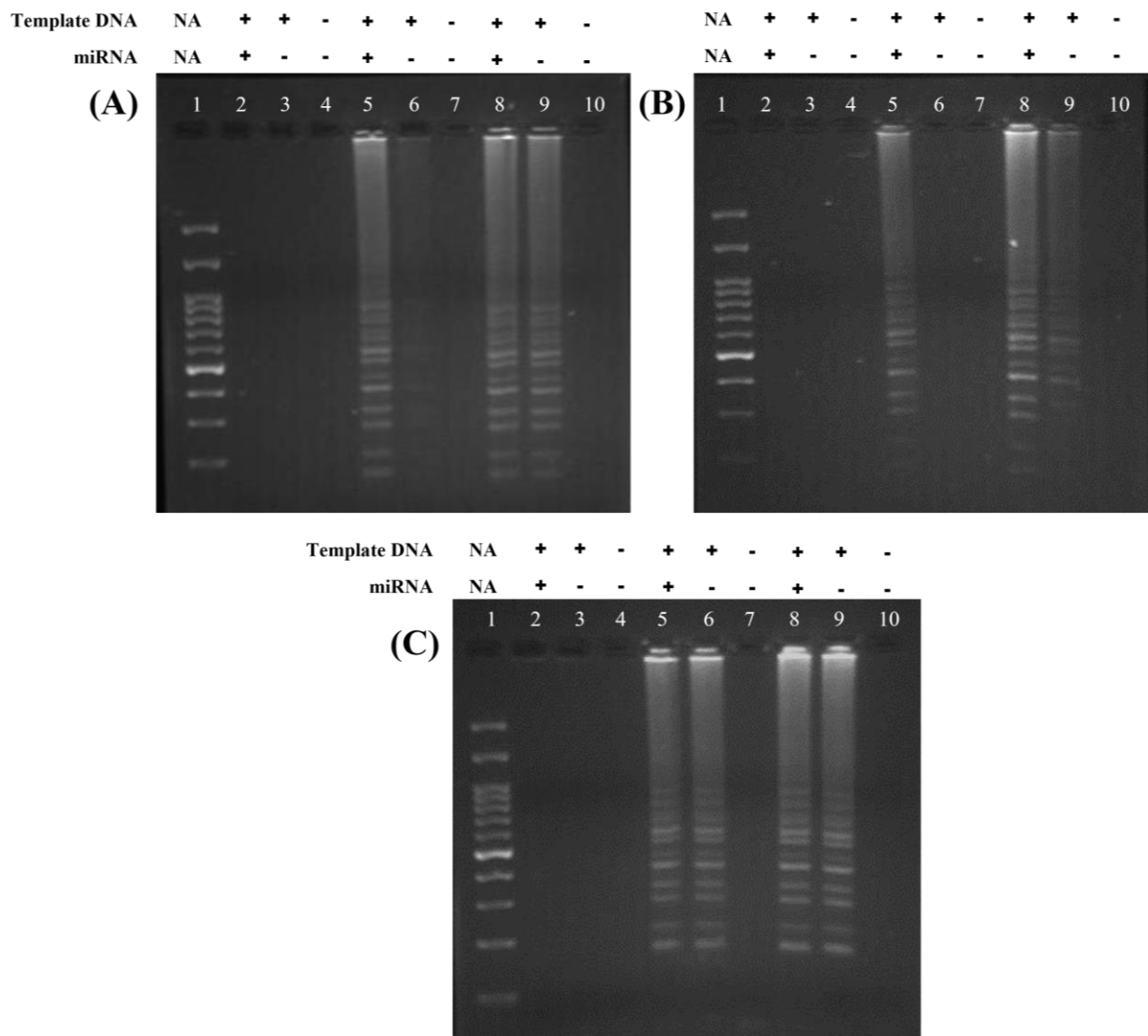


Figure 5: Optimization of one-step LAMP reaction times.

Time courses were performed for each of the three markers- (A) let-7i (B) miR-891a (C) miR144-3p. Time courses were repeated for three replicates for let-7i and miR-891a, and once for miR144-3p. Lane 1 on all panels is a 100 bp ladder. Lanes 2-4 were LAMP reactions run for 30 minutes, lanes 5-7 were run for 45 minutes, and lanes 8-10 were run for 60 minutes. Lanes 2, 5, and 8 are positive controls where 10^{11} copies/ μ L of synthetic miRNA was added to the reaction mixture. Lanes 3,6, and 9 are blanks where the reactions were prepared to include template DNA but not synthetic miRNA. Lanes 4, 7, and 10 are non-template controls with all reaction components except template DNA and miRNA. All LAMP reactions were performed at 55°C.

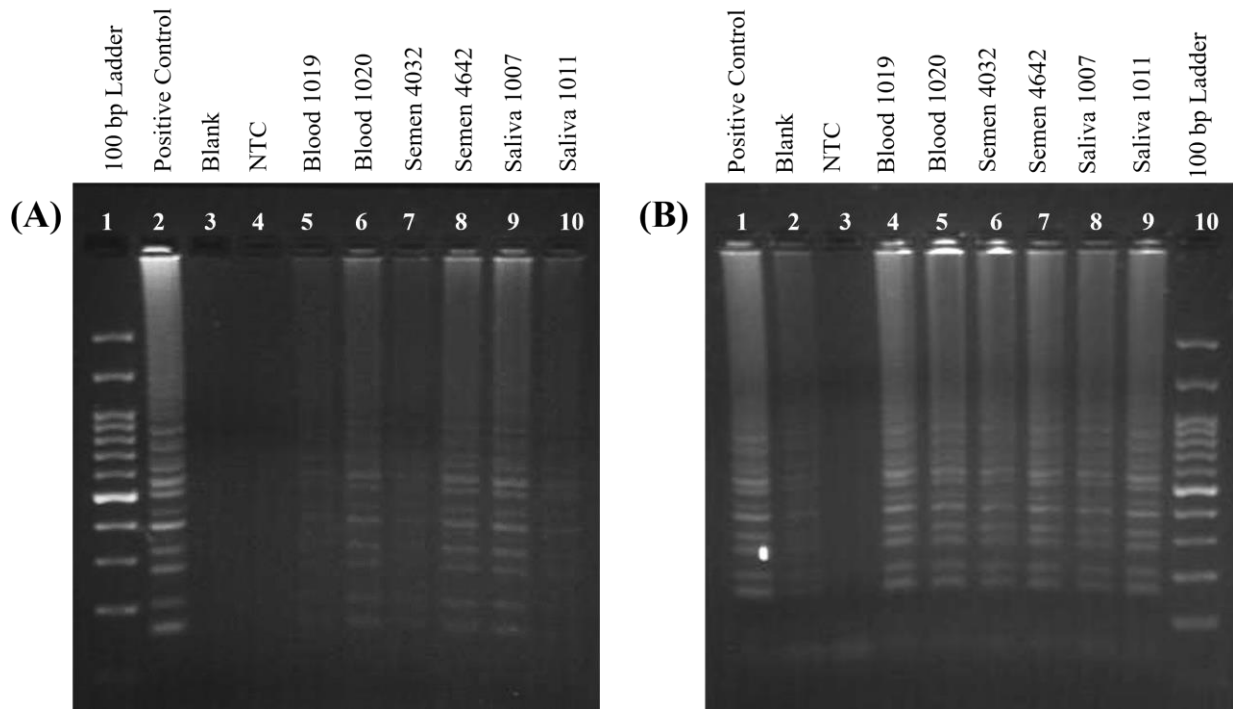


Figure 6: Evaluation of one-step LAMP detection of body fluid extracts.

Detection of (A) miR-891a and (B) let-7i in RNA extracts from blood, semen, and saliva using one-step LAMP. RNA extracts were previously extracted and named according to their anonymous donor identification number in the Forensic Science Biological Sample Registry. LAMP was performed at 55°C for 45 minutes, followed by termination at 80°C for 10 minutes. Positive controls, blanks, and NTCs were prepared as described in Figure 5. All RNA extracts were added to the LAMP reaction at a volume of 2 μ L.

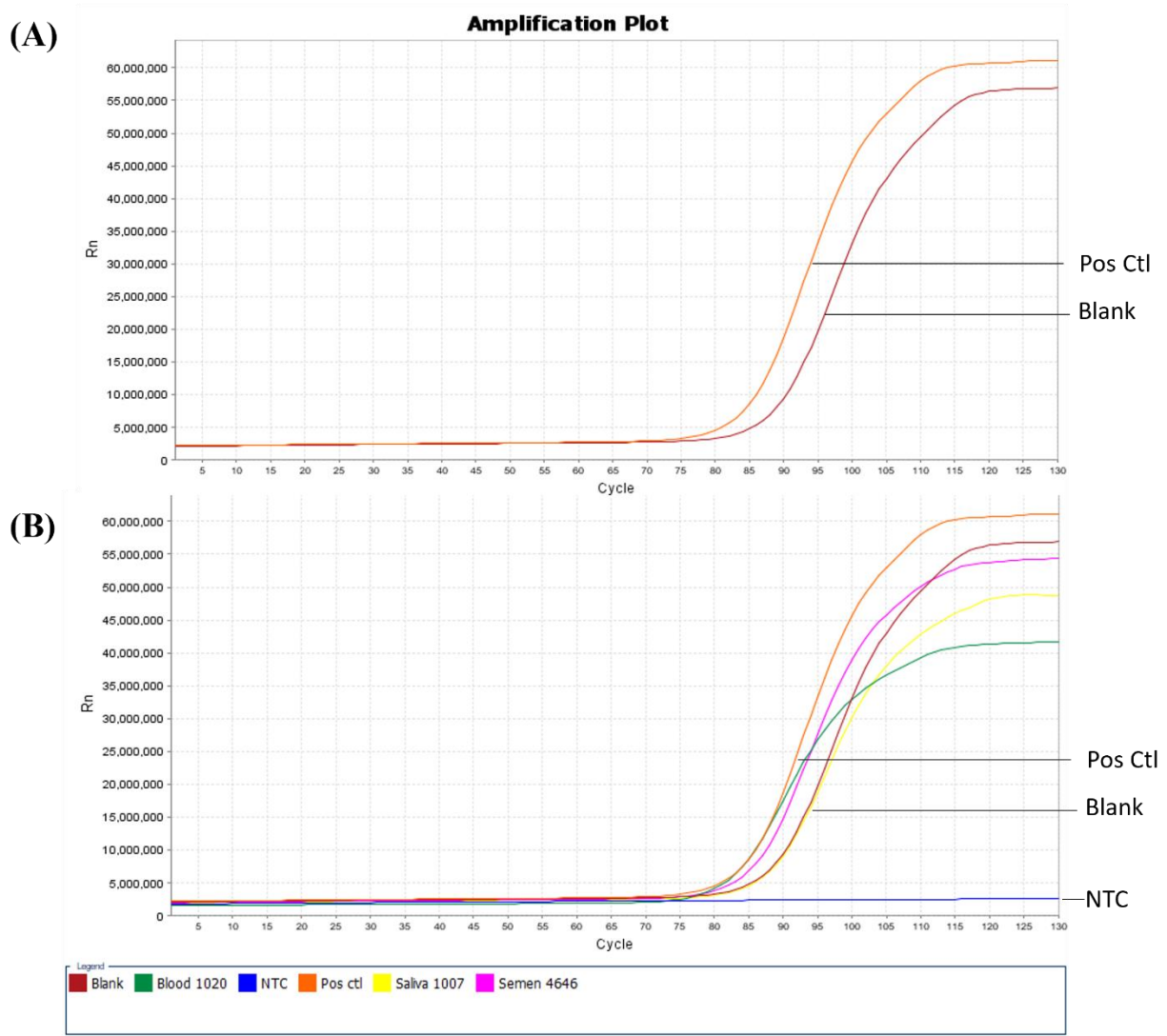


Figure 7: Real-time SYBR Green LAMP detection of let-7i.

A small sample set containing a positive control (10^6 copies of synthetic let-7i), blank, NTC, and RNA extracts from one donor each of blood, saliva, and semen was investigated. Figure 7A displays positive control and blank samples only, and Figure 7B displays all samples. LAMP was performed at 60°C for 130 30-second cycles.

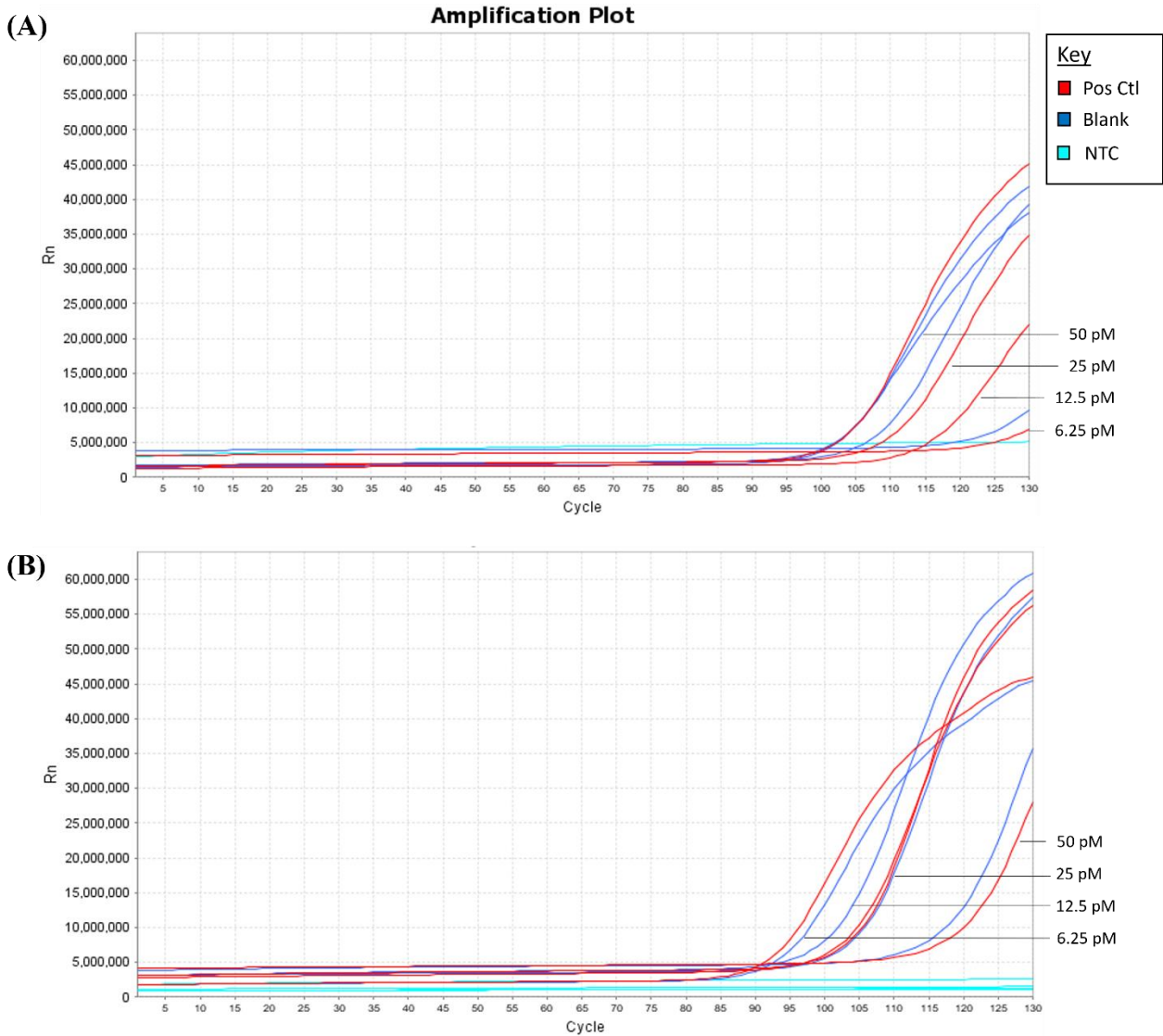


Figure 8: Real-time SYBR Green LAMP detection of *let-7i*, altering (A) template DNA concentration and (B) B3 primer concentration.

Each sample set included a positive control (10^6 copies of synthetic *let-7i*), blank, and NTC. LAMP was performed at 60°C for 130 30-second cycles.

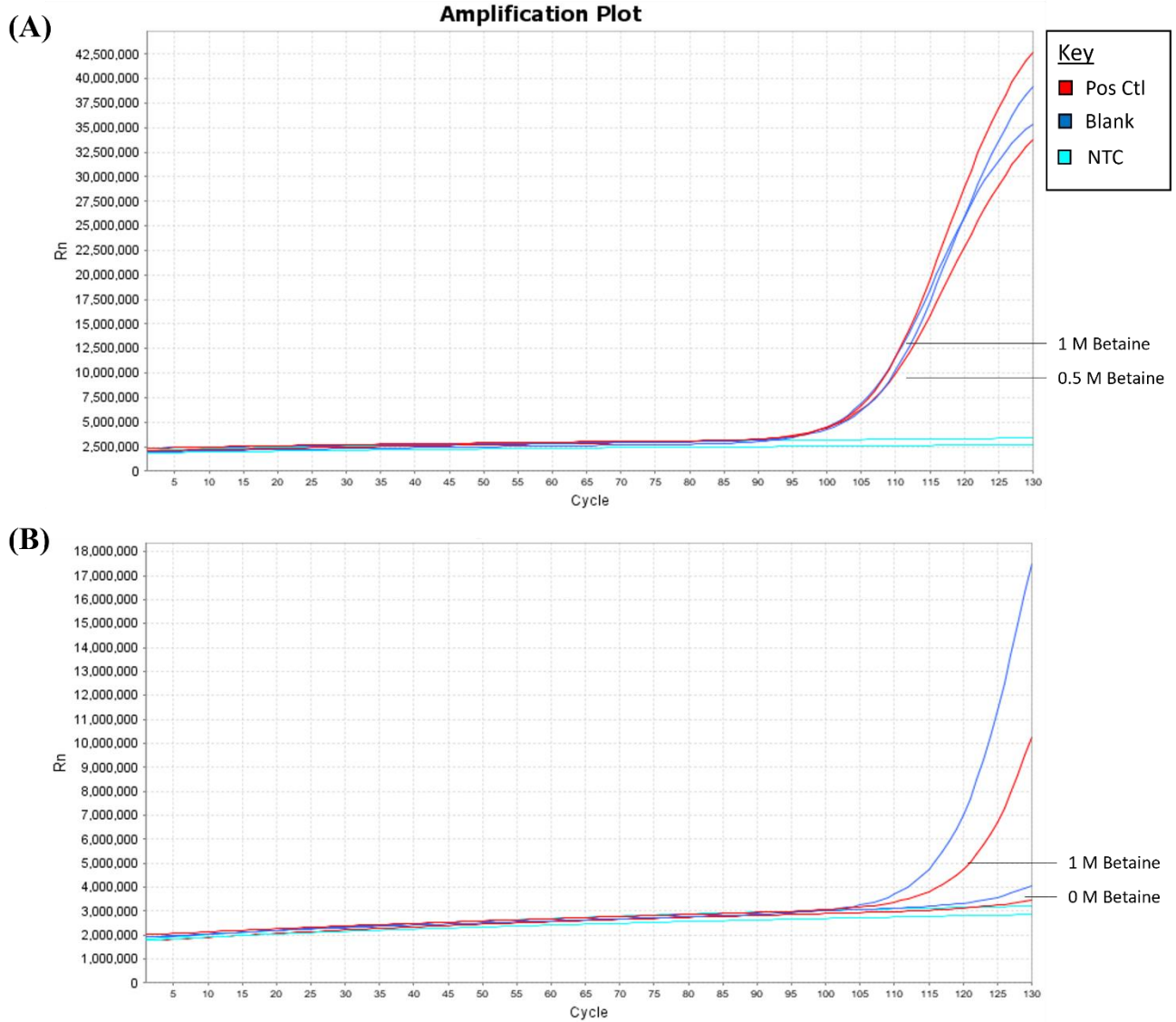


Figure 9: Real-time SYBR Green LAMP detection of let-7i investigating the effects of (A) decreasing betaine by a factor of one half or (B) removing it altogether.

Each sample set included a positive control (10^6 copies of synthetic let-7i), blank, and NTC. LAMP was performed at 60°C for 130 30-second cycles.

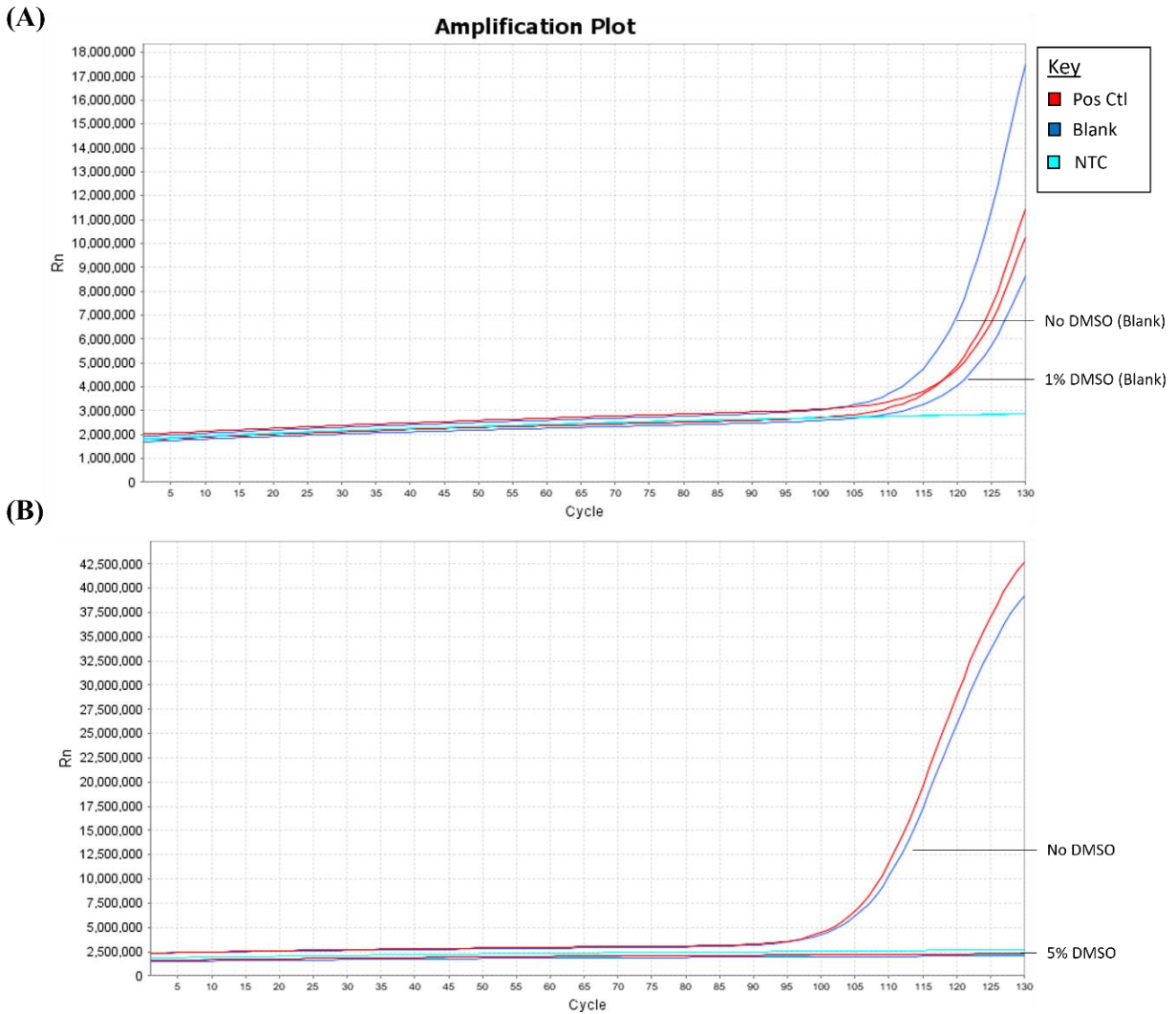


Figure 10: Real-time SYBR Green LAMP detection of let-7i investigating the effects of adding dimethylsulfoxide (DMSO) to LAMP reactions at concentrations of (A) 1% and (B) 5%.

Each sample set included a positive control (10^6 copies of synthetic let-7i), blank, and NTC. LAMP was performed at 60°C for 130 30-second cycles.

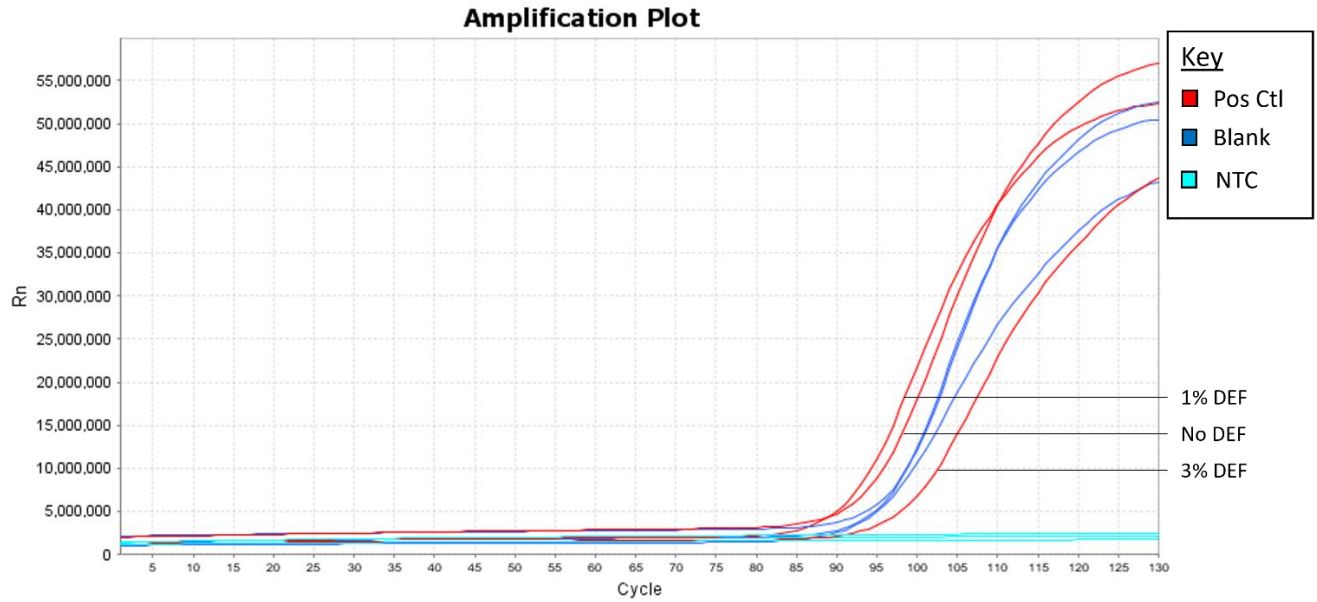


Figure 11: Real-time SYBR Green LAMP detection of let-7i investigating the effects of adding diethylformamide (DEF) to LAMP reactions at concentrations of 1% and 3% .

Each sample set included a positive control (10^6 copies of synthetic let-7i), blank, and NTC. LAMP was performed at 60°C for 130 30-second cycles.

Vita

Alisha Whelan was born and raised in Phoenix, Arizona. She graduated from the University of Arizona in 2016 with a B.S. in Molecular and Cellular Biology and a minor in Psychology. While at the University of Arizona, she worked in a molecular biology lab investigating the function and regulation of DEAD-box RNA helicases. From 2016 to 2020, Alisha worked in the life sciences department of Regenesis Biomedical Inc. researching the impacts of pulsed electromagnetic frequency therapy (PEMF) on gene expression. She joined the M.S. in Forensic Science program at Virginia Commonwealth University in 2020 and began working in the Williams laboratory shortly thereafter. Here she worked towards the development and optimization of a loop-mediated isothermal amplification (LAMP) assay for the detection of miRNAs. During her time at VCU, she also served as a lead graduate teaching assistant for the forensic science department, where she helped facilitate multiple graduate and undergraduate serology and DNA laboratory classes.