Integration of Commercial Pregnancy Test in the Point-of-Care Diagnosis of Pathogenic Nucleic Acid Biomarkers

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Abstract

The detection of nucleic acid biomarkers in point-of-care diagnostics is currently limited by technical complexity, cost, and time constraints. However, to overcome these shortcomings, a solution can potentially be realized through the combined use of isothermal nucleic acid amplification, programmable nucleic acid circuitry, and a standard pregnancy test. First, loop-mediated isothermal amplification (LAMP) of pathogenic nucleic acids can be used to specifically enhance the presence of target biomarkers found in circulating nucleic acid (CNA) samples from bodily fluids that include blood, saliva, and urine. Once amplified, the mechanics of one-step toehold-mediated strand displacement (OSD) reactions can be used to detect subsequent LAMP amplicon product. By targeting a single-stranded loop LAMP amplicon region, a controlled and complementary OSD probe can be produced such that one strand of the reporter is biotinylated and another bears the hormone hCG. In the presence of LAMP amplicons, hCG-bearing strands from the OSD probes can then be released on the basis of strand displacement activity. The subsequent changes in free-hCG can then be measured using a commercial pregnancy test. Currently, results have demonstrated the success of this diagnostic pathway using a three-way junction hCG-OSD probe to detect synthetic biomarker samples both in buffered solution and in human serum. This overarching method can thus have the potential to be applied to any nucleic acid biomarker target, offering a broad and hopeful future for its use in point-of-care and low-resource diagnostics.

Introduction

Nucleic Acid Biomarkers as Point-Of-Care Diagnostic Targets

In modern medicine, the need to develop point-of-care diagnostics can prove pivotal in the spread of healthcare to areas of low-resource and poor socioeconomic bearing. The simplistic needs of point-of-care devices limit their use to a small range of biological analytes, preventing their current implementation in the detection of certain pathogenic biomarkers. One such target that has been unfortunately blocked from the benefits of point-of-care diagnostics lies in nucleic acid biomarkers. In traditional medicine, nucleic acids are frequently used in the diagnosis of diseases that span viral, bacterial, and cancerous origins. Despite the commonality of nucleic acid biomarkers in clinical diagnostics, two issues currently hinder their viability for detection in point-of-care settings. First, nucleic acid biomarkers are often found in trace amounts throughout bodily samples. In a typical diagnostic sample from a human patient, pathogenic nucleic acids can be found in what is known as circulating nucleic acid (CNA). This reservoir of nucleic acids is a mixture of DNA and RNA that arises as a minute byproduct of cell degradation or apoptosis within the body and can be observed in fluid samples such as blood, saliva, and urine. The result, however, is the presence of a complex sample matrix, heavy dilution factors, and competing nucleic acids that can all attribute to low amounts of target nucleic acid biomarker available for detection. Fortunately, this problem can be easily overcome with appropriate resources by utilizing one of several methods available to selectively amplify target nucleic acids. Despite this, the second issue therein lies in producing a signal that is capable of reflecting the amount of amplified target nucleic acid in a product-specific manner. In most cases, nucleic acid visualization methods are infrequently target-specific (e.g: gel electrophoresis, pyrophosphate release, etc.) and thus provide signal in the presence of any nucleic acid. This provides complexity in the case of nucleic acid amplification reactions, which oftentimes generate non-specific side products which can clutter signal. To again overcome this issue, target-specific nucleic acid visualization systems exist and can be used, but rely heavily on experimentally and technologically advanced methods that require highly trained individuals and expensive machinery, such as real-time PCR. These techniques not only carry a high price tag, but are also time-consuming and lack field robustness. As a result, the capability to detect nucleic acid biomarkers is clouded by its overall complexity, cost, and timing.

With this in mind, if nucleic acid biomarkers are to be detected under point-of-care settings, it is necessary to develop or modify a new schematic of molecular diagnostics. To make this viable, the two main issues that must again be addressed are the ability to amplify target nucleic acids and the ability to produce a signal transduction method that is specific to the amplified target.

Isothermal Nucleic Acid Amplification

In considering the two issues at hand when dealing with point-of-care nucleic acid detection, the problem of amplification is perhaps better dealt with and explored. Through current advances in biotechnology, there is wide array of amplification reactions that exist for nucleic acids. Of these methods, however, the ones best suited for use in point-of-care diagnostic settings most likely lie within the range of isothermal amplification (IsoT) techniques. Unlike traditional PCR, isothermal amplification reactions do not rely on cycling temperature changes to undergo nucleic acid amplification. Instead, thermodynamic favorability from base-pairing is used in combination with DNA polymerase strand-displacement activity to drive seemingly autonomous polymerization circuits. By being held at only a single temperature, isothermal amplification reactions offer a decreased need in complex machinery used relative to traditional PCR methods. As such, isothermal amplification is typically much cheaper and less technical, matching well with the requirements of point-of-care diagnostics.

Thus, with the potential solution for dealing with trace amounts of biomarker lying within isothermal nucleic acid amplification, it is important to consider which type of isothermal reaction to use when observing downstream applications. Several isothermal reaction schemes exist, including: rolling circle amplification(RCA), strand displacement amplification (SDA), loop-mediated isothermal amplification (LAMP), and many others. Of these options, LAMP is likely the most suitable for point-of-care use as it offers increases in sensitivity, reaction speed, and amplicon yield above most other methods. Furthermore, it can be applied to non-denatured genomic DNA samples under isothermal reaction conditions, which has shown to be widely useful in genetic diagnostics and point-of-care device fabrication.

Overall, LAMP offers powerful nucleic acid detection capabilities. Under controlled conditions, it has been demonstrated to detect as few as two starting molecules within reaction volumes paralleled to standard point-of-care diagnostic samples (being within the microliter range.) The LAMP reaction involves a six-primer configuration that ultimately generates a combination of both spurious and target-specific side products (Figure 1,A&B). As is the case with any isothermal continuous amplification reaction such as LAMP, the formation of side product amplicons can provide cluttering signals that add to the difficulty in using non-target-specific amplification detection. LAMP, however, offers the benefit in that one of the amplicons that is generated as a byproduct of this reaction is a semi-duplex dumbbell DNA structure composed of two target-specific single-stranded loops (Figure 1, C). Two variants of this type of amplicon are formed for a total of four individual and distinct loop regions.

Thus, by using LAMP, the issue of low target nucleic acid biomarker concentrations is overcome as the amplification reaction can produce extensive fold-increases in product. Using LAMP also continues to maintain a point-of-care focus due to the requirement of using only a single reaction temperature. Furthermore, the single-stranded loop amplicons generated as one of the side-products of LAMP can further act as a platform for amplification detection in a target-specific manner. Combining these factors together, LAMP can promisingly help solve the first issue to point-of-care nucleic acid recognition while similarly building the foundation to address the second, more challenging concern of transducing a signal from the amplified product.

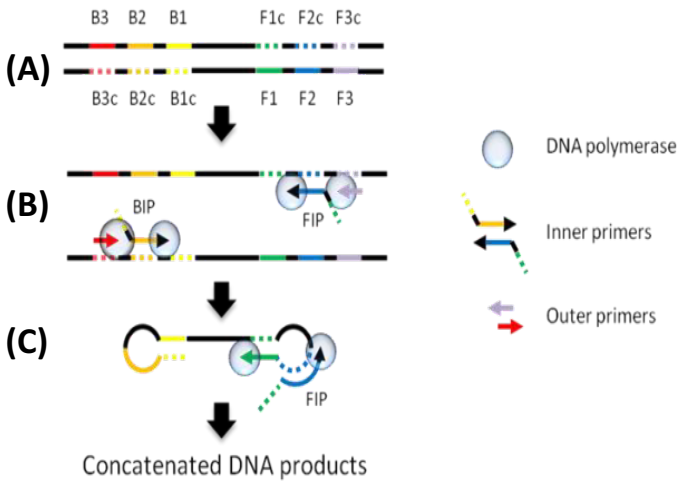

Figure 1. Generalized schematic for target LAMP amplicon production. *(A) Six-primer configuration for LAMP that is highly programmable and specific to a target gene of interest. (B) Example representation of enzyme-binding and strand-displacement activity of DNA polymerase that drives isothermal amplification at a single and constant temperature. (C) Example representation of partial-duplex dumbbell LAMP amplicon product with two target-specific loop regions. Single-stranded loops serve as scaffold for continuous amplification and are capable of binding to additional DNA.*

One-Step Toehold-Mediated Strand-Displacement

With the selection of LAMP as an amplification method, there is thus a pressing need to develop a real-time, sequence-specific, and robust method for monitoring LAMP that can readily separate true signal from non-specific noise in a point-of-care setting. One potential solution for this could come from the use of nucleic acid circuits. Nucleic acid circuits are enzyme-free systems that are synthetically designed to perform desired logic functions (such as the AND or OR functions found in computer programming.) In developing various schemes, appropriate circuit function has been shown to be dependent on the slightest defects in circuit design, including those from DNA structures or even from minute sequence differences as small as single nucleotide polymorphisms (SNPs). Most circuits are based on the idea of toehold-mediated strand displacement, in which thermodynamic favorability of base-pairing drives spontaneous exchanges of strand-binding between various single-stranded and semi-duplex nucleic acid structures (Figure 2). By consequence, this system can thus be exploited for use in the signal transduction of LAMP amplicons, which similarly bear necessary single-stranded loop regions. Sequence-specific partial-duplexed nucleic acid structures termed one-step toehold-mediate strand-displacement (OSD) probes can be created such that they bear a complementary strand to a target loop region of a LAMP amplicon. Increased complementarity of the partially-duplexed probe can then serve to selectively release a strand of less complementary nucleic acid in the presence of the loop.

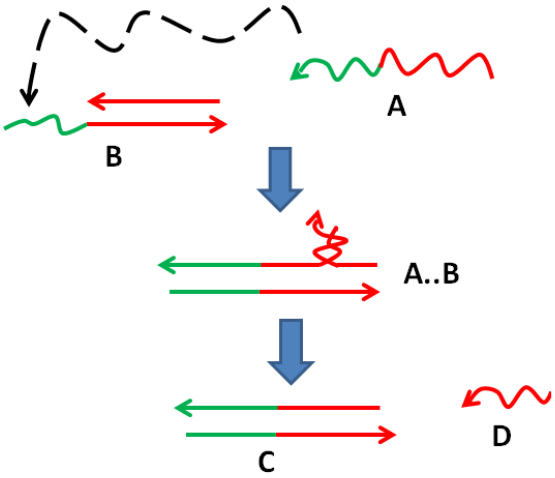

Figure 2. Toehold-mediated strand displacement reaction. *The overall reaction is A+B= C+D. 'A' is single-stranded oligonucleotide and 'B' is a semi-duplex with both a double-stranded region and an exposed single-stranded tail. Different colors represent the sequence complementarity. For example, 'A' and 'B' share the same sequence information fragment (denoted by the red domain.) The kinetics of this reaction largely depends on the toehold length, which is the number of nucleotides (denoted by the green domain) at 5' termini of component B. Increased thermodynamic favorability of the binding between 'A' and 'B' causes for the disassembly of the double stranded region of 'B'. The final product is a highly complementary duplex structure that resulted from the hybridization of 'A' and 'B,' termed 'C,' and the released fragment once hybridized to 'B,' termed 'D.'*

hCG OSD-Probes and the Commercial Pregnancy Test

While OSD probes are highly programmable and sequence-specific, the single release of a non-complementary strand of nucleic acid still does not provide enough signal transduction to visualize LAMP amplification of a target nucleic acid biomarker. As such, there is a need to further modify OSD probes such that the strand-release reaction generates a signal that

is measurable in low-resource and point-of-care settings. One potential way to achieve this could be through integration of a commercial pregnancy test into the entire detection system. For several decades, pregnancy tests have found their place on the shelves of local markets and drugstores across the world. As a diagnostic device, these tests are perhaps one of the most commonly recognized and commercially available point-of-care diagnostics available in the market to date. The popularity of this device is likely linked to the simplicity of its use. To perform its function, a typical pregnancy test is able to detect a glycoprotein hormone known as human chorionic gonadotropin (hCG), which is released in elevated levels by the placenta and is readily found in the blood and urine of pregnant women throughout the duration of pregnancy.

hCG detection in a pregnancy test is achieved by what is known as a lateral flow immunochromatographic assay. Such assays can be split into two separate components that individually contribute to the detection of a target analyte. The first section is the loading pad and is the area in which a fluid sample is administered to. Furthermore, the loading pad contains antibodies which are capable of selectively binding in excess to the target analyte and are also modified such that they are conjugated to gold nanoparticles (AuNPs). These AuNPS provide the pink coloration that can later be used to visually signal for the location of these primary anti-analyte antibodies. Further from the loading pad, the second section of the assay is the conjugate pad and is further subdivided into the test line and the control line. Similar to the loading pad, the test line portion of the conjugate pad contains anti-analyte antibodies that are capable of specifically binding to different epitopes on the target analyte relative to the primary antibody. These secondary anti-analyte antibodies on the test line, however, are conjugated to the pad itself and are thus immobilized on the strip in a single band. Further downstream from the test line is the control line section of the conjugate pad. Again, a series of antibodies are uniformly immobilized on the control line; however, these antibodies are designed to specifically bind to an inert epitope on the primary anti-antigen antibody in both the native and immunocomplexed form.

Clinical samples are added to a lateral flow assay strip to detect for the presence of certain biologically relevant analytes. This can be put into the perspective of a standard pregnancy test, in which the diagnostic biomarker analyte is hCG. In the case where a sample is added from an individual with hCG present in the body, the hormone is first able to interact and bind with the primary anti-hCG antibody which then forms the hCG-antibody immunocomplex. Capillary flow carries the mixture down the test strip where it then reaches the secondary anti-hCG antibodies that are uniformly embedded on the conjugated pad. If hCG and the subsequent immunocomplex are present, the secondary anti-hCG antibodies can capture the immunocomplexes, sandwiching the hCG between the two antibodies. As a result of this, the gold nanoparticles conjugated to the primary antibody are localized on a single band, producing the first pink "test" band on the test line. Excess primary antibodies and the remaining immunocomplexes further flow down the strip where they reach the control line. Since the control line binds to the primary antibody regardless of its binding to hCG, the second pink "control" band again forms from the localization of the AuNPs,

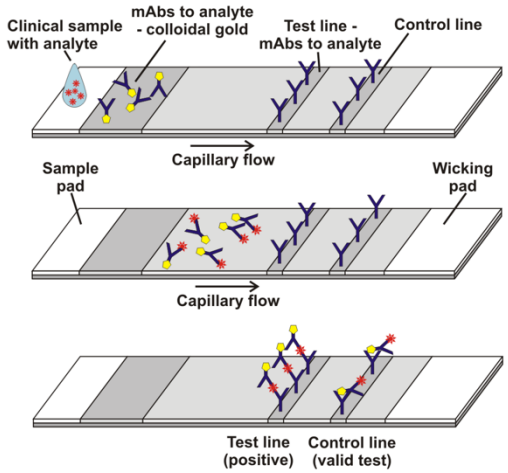

***Figure 3. Lateral flow design**. First, analytes in a fluid sample are added to the sample loading pad. Analytes form complexes with primary anti-analyte antibodies that are conjugated to gold nanoparticles. Lateral flow down the test strip brings complexes and remaining unbound antibodies to the test line, where secondary anti-analyte antibodies can again bind the analyte and localize gold nanoparticles in a single line. Continuous flow draws the sample further down the strip where antibodies capture additional primary anti-analyte antibodies and again localize gold nanoparticles in a single line.*

Immunochromatographic lateral flow assays, specifically in the case of pregnancy tests, are robust in their stability and detection ranges, further adding to their global popularity. From the simplistic pregnancy test design that is dependent only on the presence of hCG, pregnancy tests can be used to monitor the activity of any system in which there is a change in hCG release or concentration. Currently, this is limited to the hCG found in samples from pregnant women; however, this system can be adapted to the previously described OSD probes. In the OSD reaction, the strand that is released from the OSD probe in the presence of the LAMP amplicon loop is subject to any standard nucleic acid modifications. One such modification is the conjugation of the released strand to any desired protein, a feat which can be easily achieved by a variety of tagging methods. For example, a SNAP-tag can be used.

In SNAP-tag biochemistry, a protein known as human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT) is co-expressed with another protein of interest through the use of synthetic gene vectors. hAGT provides a reactive cysteine residue which is then able to transfer and form an irreversible covalent bond with alkyl or benzyl groups from substrates that include O^6-alkylguanine-DNA and O^6-benzylguanine (BG). Traditionally, this system is taken advantage of by producing fluorescent molecules conjugated to benzyl group of BG which then become covalently linked to the hAGT SNAP-tag and thus to the target protein. In modifying this system, oligonucleotides can also be synthetically produced such that desired strands are similarly bound to BG. Thus, it is possible to conjugate a specific nucleic acid strand to an equally specific by utilizing the SNAP-tag (Figure 4).

Combining these ideas together, conjugation of hCG to an OSD probe can be utilized to selectively release hCG in the presence of LAMP amplicon loops, which again corresponds to the sole amplification of a given nucleic acid biomarker. Subsequent strand-displacement of the hCG-bearing strand can then be monitored using a commercial pregnancy test, just as if the hCG in solution had been a result of hCG released by the placenta.

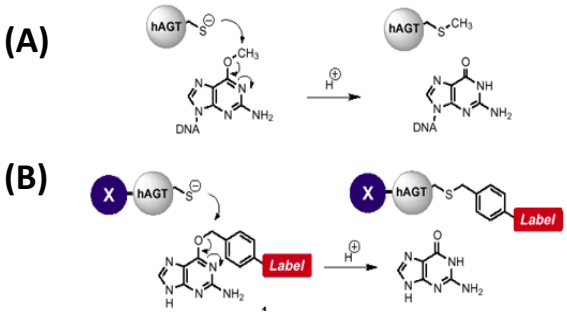

Figure 4. ***SNAP-tag reaction scheme.*** *(A) hAGT cysteine residue displacement reaction with native substrate, O^6-alkylguanine-DNA base. (B) Co-expressed target protein (labeled 'X') linked to hAGT undergoing reaction with functionalized non-native substrate, O^6-benzylguanine (BG).*

Overview

Combining these ideas together, it is possible to design a system in which LAMP is first used to specifically amplify nucleic acid biomarkers and produce characteristic loop amplicons. Next, using the principles of strand displacement, a synthetic oligonucleotide reporter probe can be produced such that the probe releases an hCG-bearing strand in the presence of target LAMP amplicons. Biotinylation of this probe can allow for its controlled localization in efforts to sequester the undesired activity of unreleased hCG. Any hCG thereby released into solution by the OSD reaction can then be ran on a pregnancy test strip in which the final readout is an easily quantifiable colorimetric signal. This system could thus potentially be used to provide yes-or-no diagnostic readings for any desired nucleic acid biomarker.

Results

Production of the diagnostic pathway began with the expression of the hCG-SNAP protein. This series of experiments was completed by the collective works of Dr. Yan Du, Dr. Arti Pothukuchy, and Jimmy Gollihar. Expression was conduced in mammalian cells. After expression and purification, final hCG-SNAP product was ran on an SDS-PAGE gel (Figure 5, A). Note that the alpha and beta bands for the hCG protein indicate the two components of the heterodimer. As indicated, the SNAP attachment was

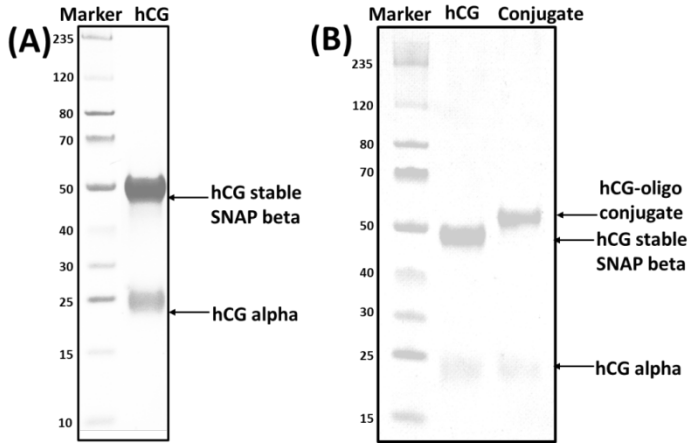

Figure 5. hCG-SNAP protein synthesis and conjugation SDS-PAGE gel verification. *(A) Initial results after synthesis and purification of the hCG-SNAP protein. (B) Side-by-side comparison of the purified hCG-SNAP protein (lane termed "hCG") and the conjugated hCG-oligonucleotide product (lane termed "Conjugate.") Figure obtained from Dr. Yan Du.*

realized specifically on the beta component. Upon successful expression, the conjugation of the hCG-SNAP protein with the benzylguanine-modified oligonucleotide of the final OSD probe was performed. Note that the nucleic acid sequences used for the eventual production of the hCG-OSD probe were specific to the Zaire Ebola virus gene biomarker ZEBOV-VP30. After purifying the conjugated product, both the unconjugated and conjugated hCG-SNAP proteins were run on an SDS-PAGE gel to verify successful binding (Figure 5, B). Additional characterization studies were performed upon completing the expression and purification processes but were excluded to focus on primary product validation experiments.

Once conjugation was complete, verification of the hCG-oligonucleotide binding capabilities with anti-hCG antibodies was conducted and compared relative to a commercial hCG without any modification (Figure 6, A&B). Testing was conducted by Dr. Yan Du. This was achieved using an enzyme-linked immunosorbent assay (ELISA) in which the capture and enzyme-linked antibodies chosen for the assay corresponded to the primary and secondary anti-hCG antibodies used in a standard pregnancy test. In observing the results from the ELISA, it is apparent that the in-house synthesized hCG-oligonucleotide conjugate had a dissociation constant (K_d) value equal to 1.0 nM of conjugated protein. Alternatively, the K_d or the commercially obtained unmodified hCG was equal to 45.5 nM of protein.

After demonstrating the binding performance of the hCG-oligonucleotide to standard anti-hCG antibodies (as would be found on a pregnancy test strip), production of the three-way junction hCG-OSD probe was conducted. The initial design for the hCG-OSD probe was a duplex structure of two partially complementary sequences in which the biotinylated strand was designed to target one of the four loop regions of the ZEBOV-VP30 LAMP amplicon and the other strand was the conjugated hCG-oligonucleotide. This probe was subsequently used in LAMP product-detection experiments. At first, increasing concentrations of a mimic LAMP target (short oligonucleotide fragments that correspond to the loop

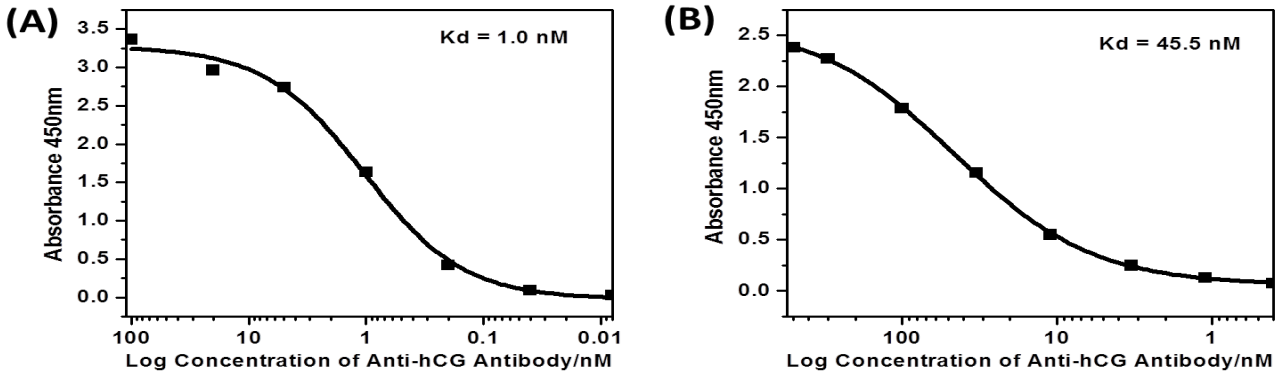

Figure 6. ELISA comparison of commercial and oligonucleotide-conjugated hCG to pregnancy test antibodies. *Dose response curves were generated for the in-house hCG-oligonucleotide conjugate (A) and a commercially purchased hCG sample (B). The corresponding K_d values were 1.0 nM and 45.5 nM, respectively. Figure obtained from Dr. Yan Du.*

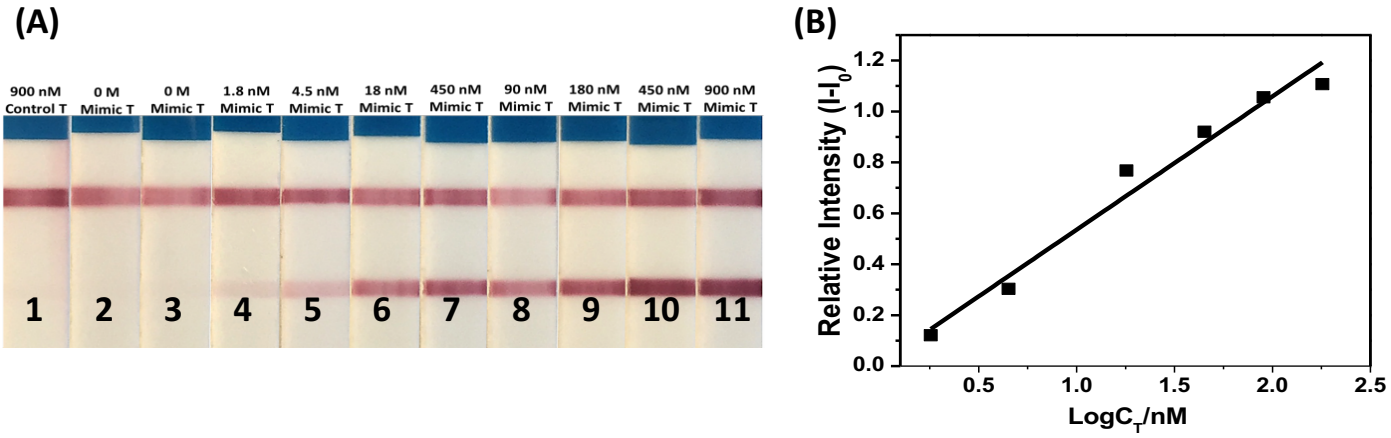

Figure 7. Pregnancy test detection using mimic LAMP targets. *(A) Raw results obtained from pregnancy test strips. Strip 1 shows high concentrations of a non-target control (Control T). Strips 2-3 show negative controls with no mimic target (Mimic T). Strips 4-11 show various concentrations of mimic target. (B) Pregnancy test intensity plot. Relative intensities were calculated based on a ratio of the control and test lines. Relative intensities were plotted against the logarithmic concentration of mimic LAMP target used. Note that the duplex hCG-OSD probe was used for this series of tests; all unreacted probes were removed prior to running on pregnancy test strips.*

region of the LAMP amplicon) were incubated with the hCG-OSD probe. After separating the sample fluid from any unreacted hCG-OSD probes through magnetic bead collecting, the subsequent hCG release was monitored using commercial pregnancy test strips (Figure 7, A). Computational quantification of the band intensities relative to the control band were then determined using Image J software. This data was used to plot the signal intensity from the pregnancy tests as a function of the concentration of mimic target used to release the hCG-bearing strand from the hCG-OSD probe (Figure 7, B).

In transitioning to the product from actual LAMP reactions on the ZEBOV-VP30 gene, it was discovered that signal for all positive controls was being quenched. As such, a new design for the OSD probe was achieved through the use of a three-way junction. In this setup, a biotinylated strand (termed P2) was partially duplexed with high complementarity to an unmodified strand (termed P3). The biotinylated strand had a sequence that was target-specific to one of the loop regions of the LAMP amplicons but also contained a non-complementary sequence attached to its end. Likewise, at the same end, the P3 strand was also tagged with a non-complementary sequence different from that of the P2 strand. The result of this was a partial duplex: one end contained a toe-hold suitable for binding to LAMP amplicons and initiating strand displacement; the other end extended two fragments of non-specific and non-complementary

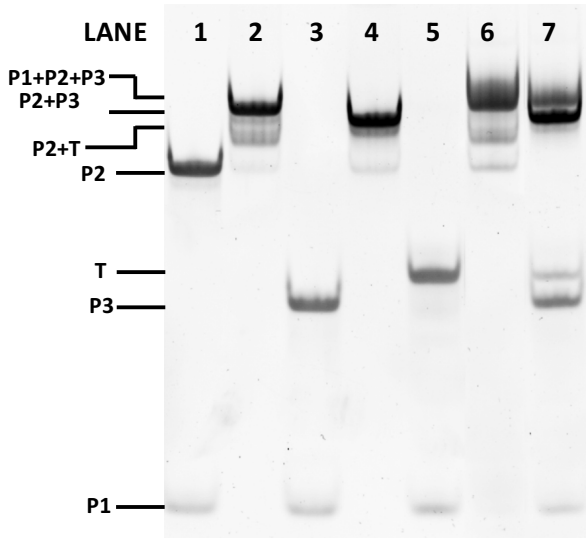

Figure 8. Native PAGE verification of three-way junction hCG-OSD probe design and release mechanisms. *The following molar additions were incubated and subsequently ran for visual analysis. Lane 1: 300 nM P1 strand and 200 nM P2 strand. Lane 2: 200 nM P2 strand and 200 nM P3 strand. Lane 3: 300 nM P1 strand and 200 nM P3 strand. Lane 4: 200 nM P2 strand and 200 nM mimic target (T). Lane 5: 300 nM P1 and 200 nM mimic target. Lane 6: 200 nM P1, P2, and P3 strands. Lane 7: 200 nM, P1, P2, and P3 strands and 200 nM target.*

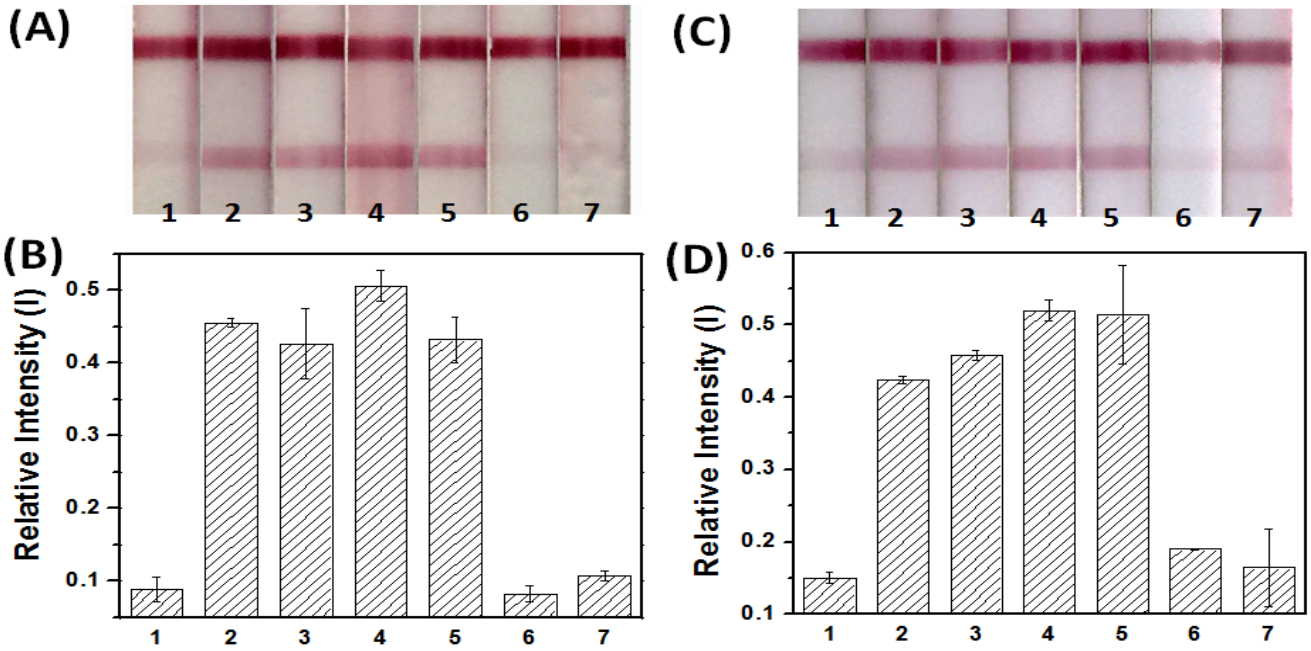

Figure 9. Pregnancy test three-way junction hCG-OSD probe detection with actual LAMP product (designed for ZEBOV-VP30 biomarker.) *(A) Raw results obtained from pregnancy test strips for LAMP reactions prepared in buffer. (B) Raw results obtained from pregnancy test strips for LAMP reactions prepared in 5% human serum. (C) Realtive intensity of pregnancy test results for LAMP reactions in buffer. (D) Realtive intensity of pregnancy test results for LAMP reactions in 5% human serum. Relative intensity calculations factored for the ratio of control lines to test lines for each strip. Strips 1: Negative control (no LAMP product). Strips 2: LAMP product from 2E1 copies of ZEBOV-VP30. Strips 3: LAMP product from 2E2 copies of ZEBOV-VP30; Strips 4: LAMP product from 2E3 copie of ZEBOV-VP30. Strips 5: LAMP product from 2E4 copies of ZEBOV-VP30; Strips 6: LAMP product of 5E6 copies of BRAF template with ZEBOV-VP30 LAMP primers; Strips 7: LAMP product from 5E6 copies of BRAF template with BRAF LAMP primers.*

oligonucleotide tags that could thus serve to bind a third strand. As such, this final strand was a newly synthesized hCG-conjugated oligonucleotide (termed P1) that had a complementary sequence to the random tags of P2 and P3, thereby forming a three-way junction (Figure 10). Since the random tag added to the end of P2 was much shorter than that of P3, there was minimal binding between P1 and P2, thus allowing P3 to serve mostly as the linker in this novel probe design. With this system, binding of the P2 strand to a LAMP amplicon was expected to cause for complete disassembly of the junction.

Once developed, verification of the three-way junction hCG-OSD probe formation was achieved using a native PAGE gel in which samples were not denatured prior to running (Figure 8). Furthermore, the appropriate hCG-release mechanism was also tested using short synthetic oligonucleotide fragments that mimicked the loop region of the LAMP amplicon product targeted by the hCG-OSD probe. In Lane 1, the hCG-bearing P1 strand and magnetic bead-bearing P2 strand were incubated. In Lane 2, P2 and the connecting strand P3 were incubated. In Lane 3, P1 and P3 were incubated. In Lane 4, the mimic LAMP target and P2 were incubated. In Lane 5, the mimic target and P1 were incubated. In Lane 6, all three components of the three-way junction hCG-OSD probe were incubated: P1, P2, and P3. In Lane 7, all three components of the three-way junction hCG-OSD probe and the mimic LAMP target were incubated.

After ensuring the successful formation and function of the three-way junction hCG-OSD probe, actual LAMP product was produced and used for further experiments. This was done by performing LAMP reactions directly on copies of the ZEBOV-VP30 gene. To do so, LAMP primers designed specifically for the ZEBOV-VP30 gene were used. The amount of initial copies of nucleic acid biomarkers used for the LAMP reactions ranged from 20 to 20,000 molecules. In addition to this, two controls were also performed by attempting the LAMP and OSD reactions on 500,000 initial copies of the melanoma biomarker

gene BRAF. The first control utilized LAMP primers specific to the ZEBOV-VP30 gene whereas the second utilized primers specific to the BRAF gene. For all samples, including both controls, hCG-OSD probes specific to the ZEBOV-VP30 gene were added and allowed to incubate. After doing so, unreacted probes were collected using a magnetic separator and the remaining samples were run on individual pregnancy test strips. Band intensity was again determined for the LAMP reactions. This entire process was conducted for LAMP reactions both in buffer (Figure 9, A&B) and in 5% human serum (Figure 9, C&D).

Discussion

While additional characterization studies were conducted to asses the expression and purification quality of the hCG-SNAP protein, the overall success of the conjugation reaction between the synthesized hormone and the BG-modified P1 strand of the final OSD probe was demonstrated from the PAGE gel (Figure 5, A&B). This was assumed from the observed increase in molecular weight corresponding to the weight of the BG-oligonucleotide for the band of the beta-segment of the hCG protein. This can be further understood from the constant molecular weight of the alpha-segment of the protein, as the alpha fragment did not have a SNAP-tag co-expressed.

Once protein expression and conjugation was successfully verified, the binding capabilities of the hCG-oligonucleotide to the antibodies found in a standard pregnancy test then needed to be checked. From the ELISA, it was demonstrated that the K_d decreased for hCG-antibody binding when comparing the conjugated hCG-oligonucleotide relative to the unmodified hCG protein (Figure 6). While the decrease in K_d indicated improved binding favorability of the hCG-oligonucleotide to the pregnancy test strip antibodies, the difference in K_d values spanned only a single order of magnitude. As such, the slights variants in K_d were considered beneficial but relatively insignificant. More importantly, however, it was determined that the K_d had not been worsened for hCG binding as a result of conjugation with the oligonucleotide. This thereby allowed for the hCG-oligonucleotide to act as a viable reporter.

Using the initial duplexed hCG-OSD probe design, it was then appropriate to determine the ability of pregnancy tests to monitor the capability of the probes to release hCG in the presence of its target. By incubating various concentrations of the mimic LAMP product with the hCG-OSD probe and running subsequent samples on pregnancy test strips (Figure 7, A&B), it can be shown that a direct linear relationship existed between the logarithmic concentration of LAMP target and the subsequent relative band intensity of the pregnancy test strip. Since hCG-release is directly correlated to the concentration of LAMP target, in the range of sample concentrations being tested, it was expected that an exponential increase in pregnancy test signal would exist as a function of the mimic LAMP product concentration. This is characteristic for lateral flow assays in the detection of their target analytes. As such, since this trend was observed, it can be inferred that the hCG-releasing system was successfully achieved in the presence of mimic LAMP targets and that this signal was capable of being monitored using pregnancy tests.

When transitioning from the mimic LAMP target to actual LAMP product, it was eventually discovered that no positive LAMP controls produced a measurable signals on any pregnancy test strips, contrary to the expectation. In reviewing the OSD design, it was discovered that the duplex hCG-OSD probe was still capable of releasing the hCG-bearing strand in the presence of the desired loop amplicon from actual LAMP product; however, it was determined that the released hCG-oligonucleotide was then binding to a different single-stranded loop amplicon. This was thought because the LAMP amplification process produces four distinct loop regions that are two pairs of sequence complements. As such, since the biotinylated strand of the duplexed hCG-OSD probe is complementary to one of the loop targets, the released hCG-oligonucleotide strand was also complementary to a different loop target. It was thus hypothesized that binding of the hCG-oligonucleotide to additional LAMP product caused for the formation of a complex that was then too large to flow down the pregnancy test strip, thereby leading to the lack of presumed signal. Thus, it was necessary to design a new OSD probe in which the hCG-bearing

strand had no sequence complementarity to any LAMP product. This was achieved using the three-way junction design, which was then necessary to verify in terms of its structure and hCG-release capabilities. To do so, various combinations of the three-way junction hCG-OSD probe components and mimic LAMP product target were incubated together and observed on a native PAGE gel (Figure 8). Based on the results, several insights into the design of the three-way junction hCG-OSD reporter were revealed. As expected, from Lanes 1, 2, 3, and 6, it was showed that strands P1 and P2 were not able to form a stable complex without the presence of strand P3. Furthermore, from Lane 4 and 5, it was demonstrated that the strand of complementarity to the LAMP target was the P2 strand, which was synthesized to bear the biotin modification. Lastly, in comparing Lane 6 to Lane 7, it was shown that there was significantly release of the hCG-bearing P1 strand in the presence of the hCG-OSD probe and mimic LAMP product.

Confirmation with mimic LAMP targets then allowed for future experiments using the product of actual LAMP reactions on desired nucleic acid biomarker samples. In analyzing the results obtained from such experiments, it was observed that the three-way junction hCG-OSD probe was able to successfully produce a measurable signal on pregnancy test strips for as low as twenty starting molecules of target gene biomarker after undergoing the LAMP reaction (Figure 9). This was appreciable in LAMP reactions conducted both in buffer and in 5% human serum. In both cases, background signal from the negative control (using LAMP reactions conducted with no initial template) showed significantly lower signals from those with the appropriate ZEBOV-VP30 gene. Furthermore, both the LAMP and OSD selectivity was demonstrated using the substitute BRAF target in which no appreciable signals were produced in the absence of either the correct LAMP primers or OSD probe. In these control samples, the background signal produced was within similar ranges to those of the negative controls, indicating weak competitive signals from non-target genes. Signal differences between buffer and serum experiments were minimal, thereby showing the low influence of a biologically-active background matrix on both the capability to perform LAMP and the ability for the three-way junction hCG-OSD probe to function.

Conclusion

Overall, from the results of this series of experiments, each step of the diagnostic pathway was synthesized and verified. It is evident that the system is successful in which a standard pregnancy test is capable of monitoring hCG-release from an OSD probe that is designed to specifically respond to LAMP amplicons produced from trace amounts of a target nucleic acid biomarker gene. While this was demonstrated using the ZEBOV-VP30 gene, with slight modifications to adjust for various sequences and different thermodynamic favorability, the potential exists for this method to be applied to an immense range of pathological conditions in which nucleic acids serve as the primary biomarker. This diagnostic technique thus offers a highly programmable, robust, and target-specific method for detecting desired nucleic acids. The simplicity of its design and its integration with a standard pregnancy test allows for a user-friendly interface that could easily be compounded into a single diagnostic device that requires only sample input to function. Overall detection time could be

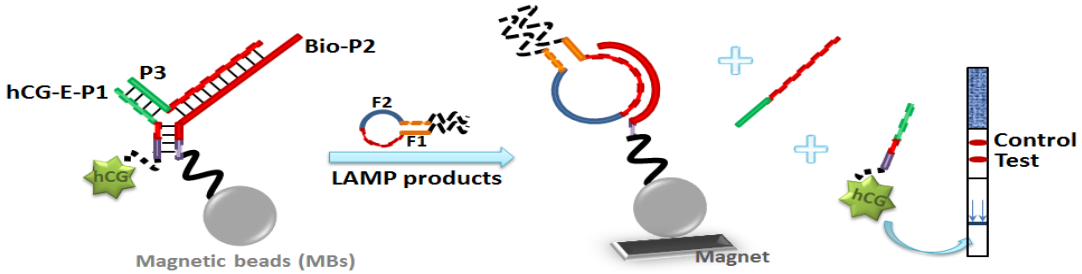

Figure 9. Overall schematic of nucleic acid biomarker detection. *Initially, LAMP product is generated for target biomarkers using a primer sequence specific to the gene. Characteristic loop-regions of a LAMP amplicon are then detected using a three-way junction hCG-OSD probe that releases the hCG-bearing strand in the presence of the amplicons. Collection of residual unreacted probes and the side-products of reacted probe creates a solution of free-hCG that can then be monitored using a pregnancy test strip.*

reduced to a proposed thirty-minute window from sample input to result readout. Furthermore, the cost of manufacturing such devices is projected to be low enough to allow for widespread use in virtually all areas of the world. Because of these qualities, the described method functions as an ideal tool for both point-of-care and low-resource diagnostics.

Future directions involve the experimentation of the aforementioned downstream goals. This includes optimization of reaction kinetics, times, temperatures, and reagents for ultimate integration into a single device. Additionally, the design of the three-way junction OSD probe must also be further investigated to achieve results such as the discrimination of SNPs. Beyond this, the robustness of the three-way junction hCG-OSD probe under lyophilized conditions must also be verified to ensure the long-term stability of the probe as well as its ability to be used in a diverse span of environmental conditions. Once all reaction functionality is verified and optimized using *in vitro* samples, future testing can then be conducted using samples obtained from patients with actual diseases of interest. After completing the remainder of these experiments, the production of a finalized diagnostic device could then be realized. In doing so, the diagnosis of nucleic acid biomarkers may be extensively improved, universally impacting global healthcare.

Methods

All chemicals were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. All enzymes and related buffers were purchased from New England Biolabs (NEB, Ipswich, MA, USA). AccuMed ® Pregnancy (HCG) Test Strips were ontained and ordered through Amazon. Methods are only briefly highlighted to demonstrate general practices implemented throughout experiments. Full details are further excluded for the purpose of simplicity. Also note that only experiments essential to the diagnostic pathway creation or repetitive validation were mentioned.

hCG SNAP-Protein Expression

Production of the hCG SNAP-Protein was achieved largely through the collaborative works of Dr. Yan Du, Dr. Arti Pothukuchy, and Jimmy D. Gollihar of the Ellington Laboratory. First, a gene vector was prepared which contained the sequence for both components of hCG. Attached to this vector was the gene encoding for a SNAP-tag. Vector transformation and subsequent protein expression in a mammalian cell culture resulted in the production of the SNAP-labeled hCG. This protein was purified from the cell culture lysate and was utilized in future experiments. Verification of successful expression was also conducted entirely by the works of Dr. Yan Du.

hCG-Oligonucleotide Conjugation and Three-Way Junction hCG-OSD Probe Production

Production of the conjugated hCG-oligonucleotide, later used in the hCG-OSD probe, was achieved largely through the collaborative works of Dr. Yan Du and Dr. Arti Pothukuchy. All oligonucleotide components were designed using PrimerExplorerV4 (Eiken, Japan) software and were all ordered through Integrated DNA Technology (IDT, Coralville, IA, USA). Future hCG-bearing oligonucleotides strands were ordered specifically with a benzylguanine-tag (BG-tag) modification. Incubation of these oligonucleotides in excess with the synthesized hCG-SNAP proteins resulted in the covalent linkage of the two. Purification of the conjugated hCG-oligonucleotide from the unconjugated hCG-SNAP protein was achieved using fast-performance liquid chromatography (FPLC). For the three-way junction hCG-OSD probe, the remainder of the production was achieved by first incubating the biotinylated P2 strand with magnetic streptavidin beads. This was conducted on a rotator at room temperature for thirty minutes. During this time, the biotin and streptavidin beads were allowed to hybridize. A magnetic separator was used to collect the hybridized structure and was washed several times with high volumes of PBS buffer. After this, the final three-way junction hCG-OSD probe was assembled by adding the remaining two strands, P1 and P3, to the sample and then allowing the mixture to incubate for 1.5 hours at room temperature on a rotator. The constructed probes were again collected with a magnetic separator and washed several times with PBS buffer. The final hybridized three-way junction hCG-

OSD probe sample was resuspended in PBS buffer and stored at 4ºC for future use.

LAMP Reaction Preparation

Mixtures were prepared to contain the following reagents: different copies of nucleic acid biomarker templates of clinical relevance (Ebola virus, melanoma cells, etc.), all six primers necessary for the LAMP reaction, betaine, $MgCl_2$ solution, and dNTPs. Note that the nucleic acid biomarkers were synthetically produced but were identical to sequences found in patient samples. Depending on the experimental constraint, certain samples were tested in solutions containing human serum, saliva, or other biological matricies. Samples were heated to denature any undesired secondary structures and to treat for any contamination. After heating, samples were followed with a chilling on ice to restore ambient temperatures. Upon cooling, *Bst* DNA polymerase 2.0 was added to each sample to initiate the LAMP reaction. Total reaction volumes were held at 30 µL in Isothermal Buffer. Samples were incubated for 1.5 hour in a thermal cycler maintained at 65 °C. Once complete, samples were briefly heated to 80ºC to heat-kill the *Bst* DNA polymerase, thus halting the reaction. Terminated LAMP reactions were stored at 4ºC until needed for future experiments. Note that all LAMP reactions were performed to completion, meaning that reactions were conducted until all available primers were depleted. As such, it was expected that final LAMP product concentration would be equal in all samples, as product concentration was dependent only on reagent availability and was independent of initial target amount.

Strand Displacement Reactions and Pregnancy Strip Testing

hCG-OSD probes hybridized to magnetic beads were washed prior to use and were resuspended in samples of interest (either solutions of mimic LAMP amplicon oligonucleotides or the product of actual LAMP reactions.) These samples were allowed to incubate on a rotator for thirty minutes at room temperature. During this time, any strand-displacement of the hCG-bearing strand was able to occur. Upon incubation completion, a magnetic separator was used to retract and condense any remaining hCG-OSD probes. The residual solution, containing any free hCG, was removed and set aside in new tubes for subsequent testing. Pregnancy test strips were directly dipped into samples and were allowed to develop for around two minutes after lateral flow was complete. Standard cell-phone cameras were used to photograph the resulting bands that formed on pregnancy test strips. Image J (NIH, USA) software was used to quantify the intensity of the bands.

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