Coupling Sensitive Nucleic Acid Amplification with Commercial Pregnancy Test Strips

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Abstract: The detection of nucleic acid biomarkers for point-of-care (POC) diagnostics is currently limited by technical complexity, cost, and time constraints. To overcome these shortcomings, we have combined loop-mediated isothermal amplification (LAMP), programmable toehold-mediated strand-exchange signal transduction, and standard pregnancy test strips. The incorporation of an engineered hCG–SNAP fusion reporter protein (human chorionic gonadotropin-O6-alkylguanine-DNA alkyltransferase) led to LAMP-to-hCG signal transduction on low-cost, commercially available pregnancy test strips. Our assay reliably detected as few as 20 copies of Ebola virus templates in both human serum and saliva and could be adapted to distinguish a common melanoma-associated SNP allele (BRAF V600E) from the wild-type sequence. The methods described are completely generalizable to many nucleic acid biomarkers, and could be adapted to provide POC diagnostics for a range of pathogens.

With advancements in medical diagnostics, it has become increasingly possible to quickly and accurately diagnose multiple disease states using nucleic acid or protein biomarkers. However, these methods generally require complex instrumentation and are often restricted to medical diagnostic laboratories. Accurate, reliable assay methods that can be used in diverse or resource-limited locations are attractive alternatives for the early identification of pathogens. Only a few point-of-care (POC) products are commercially available, but these are potentially of great utility for establishing and expanding new diagnostics approaches.

As an example, the already successful personal glucose meter (PGM) was recently modified to quantify non-glucose targets via aptamer- or DNAzyme-mediated conformational changes or sequence-specific strand exchange that in turn leads to the release of invertase. Our laboratory has further enhanced the sensitivity of nucleic acid analyte detection with PGMs by coupling loop-mediated isothermal amplification (LAMP) reactions to strand-exchange signal transduction.[13,14] Unfortunately, the use of glucometers in POC diagnostics is problematic for many biomarkers because blood or urine samples are often infused with background glucose.

These limitations have inspired us to attempt a more generalizable approach to molecular diagnostics in which we adapt isothermal amplification to commercially available and easy-to-use pregnancy test strips.[15] The key innovation allowing signal readouts to be readily adapted from one off-the-shelf device (PGM) to another very different one (pregnancy test kits) is once again the remarkable plasticity of strand-exchange signal transduction. The human chorionic gonadotropin (hCG) analyte that is already sensitively detected by commercial pregnancy test kits was site-specifically conjugated to a DNA oligonucleotide, thereby allowing signal transduction via strand exchange into both capture (signal-off) and release (signal-on) of hCG in the context of the lateral-flow dipstick (LFD) assay. The reagents proved to be stable for months, making this an excellent option for POC diagnostics in low-resource settings. While LFD assays have previously been adapted to sequence detection,[16,17] the use of strand-exchange probes has greatly increased the sensitivity, specificity, and reliability of detection.

Adapting an isothermal amplification assay (LAMP) to detection using common pregnancy test strips consisted of three steps (Scheme 1): 1) LAMP amplification; 2) capture of a hCG–DNA conjugate by the LAMP amplicon; and 3) read-out using a pregnancy test strip. LAMP produces concatemeric amplicons that contain multiple different single-strand loop sequences (between F2 and F1 (F-Loop), F2c and F1c (Fc-Loop), B2 and B1 (B-Loop), and B2c and B1c (Bc-Loop)) because the LAMP amplicon is too large to migrate in the lateral-flow test strip, capture of the hCG–oligo conjugate by one or more of these loop sequences (herein the loop between F2c and F1c, Fc-Loop) results in a loss of signal, which can in turn be interpreted as a positive detection of the nucleic acid analyte. Additional variations on this robust theme are also shown, in which strand-exchange reactions discriminate between single nucleotide polymorphisms (SNPs) of Scheme 1B) or produce positive signals (Scheme 1C). Each of these variants could be readily implemented in the overall simplistic context of applying molecular amplification reactions to a pregnancy test strip.

To implement this general strategy for antigen detection on a pregnancy test strip we needed to site-specifically conjugate DNA to hCG. We therefore designed a series of C-terminal hCG– and hCGβ–SNAP-tag fusion proteins,
expressed them in Expi293 cells, and tested their ability to bind a panel of commercial pregnancy test strips. The C-terminal fusion of the thermostable SNAP-tag to hCG provided the most robust signal in our initial screening, and all further assay optimizations were conducted with this fusion (hCG\textsubscript{b}-SNAP) (Figure S1, in the Supporting Information (SI)). A further assay of the fusion protein with ELISA revealed that the hCG–SNAP protein had an EC\textsubscript{50} (half maximal effective concentration) value of 1.0 n\text{m}, while the EC\textsubscript{50} value for commercially obtained unmodified hCG was 46 n\text{m} (Figure S2 in the SI). The hCG\textsubscript{b}–SNAP fusion could be labeled with a benzylguanine-labeled oligonucleotide with >90% efficiency, and purification using anion exchange chromatography eliminated unlabeled fusions (Figure S3 in the SI). Probe stability was assessed following lyophilization and storage at room temperature. A particularly robust probe for the Ebola virus was stored for at least 90 days without large signal deviations (Figure S4 in the SI).

We then attempted to determine if yes/no signal-off detection could be reliably obtained for important biomedical targets such as the genome of the Zaire Ebola virus VP30 (ZEBOV) and the melanoma-related oncogene biomarker BRAF. For the direct-hybridization assay (Scheme 1A), in a series of tubes 10 \mu L aliquots of hCG–P1 probe (18 nm, designed for ZEBOV LAMP amplicons; all sequences described in Table S1 in the SI) or hCG–P2 probe (8 nm, designed for BRAF LAMP amplicons) were mixed with 30 \mu L LAMP products. The hybridization reactions proceeded for 35 min at 25°C. Subsequently, the pregnancy test strips were dipped into the solutions and pictures were taken after 2 min by a cell phone. The LAMP amplicons cannot migrate on the strip after hybridization to the hCG–oligo probes, and thus only a single control line is obtained for a positive result (positive control, PC). Products that do not contain target sequence (negative controls, NCs) should not hybridize with the hCG–DNA probes, which can then flow to the test line. Thus, the appearance of signals at both the test and control lines indicate a negative result. Using the strategy described in Scheme 1A, we could definitively detect synthetic templates for these targets (Figure 1A and B). Further, following optimization of probe concentration (Figure S5 in the SI) and hybridization times (Figure S6 in the SI) we could detect as few as 20 copies of the ZEBOV templates and 2 \times 10^3 copies of the BRAF templates. The ZEBOV template spiked into human saliva (15%; Figure 2A) and human serum (10%; Figure 2B) also gave definitive results without any loss in signal intensity or specificity, even without pretreatment. Electrophoretic analyses of comparable reac-
The V600E BRAF (SNP-BRAF) T-to-A transversion mutation in the loop region were used to discriminate wild-type and mutant alleles. In parallel, hCG-labeled OSD reporters (hCG—WT and hCG—SNP) were designed that would be activated by the amplified WT- and SNP-BRAF loop sequences, respectively. LAMP-OSD-to-hCG strip transduction was carried out with 0 and 2 × 10^6 copies of the WT-BRAF gene and SNP-BRAF. When the hCG—WT reporter was introduced, no band appeared on the test line for the WT-BRAF LAMP amplicons, as expected (Figure 3). In contrast, when the hCG—SNP reporter specific for the V600E allele was used, the mutant gene could be detected.

To achieve “signal-on” detection (a target shows a positive pregnancy-test-kit result) a new strand-exchange probe was designed. The hCG conjugate (hCG—P6) was hybridized to a mediator strand (termed P8) to create a hemiduplex that could orderly be hybridized to a biotinylated strand (termed P7), creating a three-way junction (3WJ). The 3WJ was immobilized on streptavidin-modified magnetic beads (Scheme 1C). A toehold region on P7 can hybridize to LAMP products via the target-specific loop region between F1 and F2 (F-loop) and strand-displace the P6:P8 hemiduplex. The released toehold region on P8 can hybridize to its complement on the Fe-loop and thus displace the hCG—P6 reporter, allowing it to enter the lateral-flow strip (Figure S8 in the SI).

In order to demonstrate the potential utility of this design, we attempted to detect surrogate Ebola virus (ZEBOV) amplicons. Solutions of LAMP reactions (30 μL) were mixed with 1 μL loaded magnetic beads (0.5 mg mL⁻¹) and incubated for 30 min before magnetic separation, and the supernatant was run on individual pregnancy test strips. Figure 4A and 4B show a positive signal compared with negative controls (isothermal amplification buffer without ZEBOV templates, WT-BRAF templates with ZEBOV primers, and noncognate WT-BRAF LAMP products). Remarkably, as few as 20 copies of ZEBOV produced an obvious, easy-to-read positive signal on the test line of the pregnancy test strips. This was true even when the entire process was conducted with LAMP reactions in 5% human serum (Figure 4C and D).

We have coupled isothermal amplification assays to strand-exchange nucleic acid transducers and thereby generated hCG signals for direct interpretation with off-the-shelf probes. A hCG strip responds to both WT- and SNP-BRAF templates with either hCG—WT or hCG—SNP reporters. B) Quantitation of (A) using Image J software.

Figure 1. Sensitive and specific detection of ZEBOV DNA plasmid and WT-BRAF PCR product using LAMP-to-hCG transduction. The LAMP reaction proceeded at 65°C for 1.5 hours. A) The hCG strip (inset) responses to negative control without template (strip 1), to different amounts of ZEBOV templates (strip 2: 20 copies; strips 3: 200 copies; strip 4: 2 × 10^3 copies; strip 5: 2 × 10^4 copies; strip 6: 2 × 10^5 copies), to side products (strip 7), and to nontarget WT-BRAF LAMP products (strip 8) are quantitated. Strip 0 is hCG—P1 in 1× iso buffer. B) The hCG strip (inset) responses to negative control without template (strip 1), to different amounts of WT-BRAF templates (strip 2: 2 × 10^3 copies; strip 3: 2 × 10^4 copies; strip 4: 2 × 10^5 copies), to side products (strip 5), and to nontarget ZEBOV LAMP products (strip 6) are quantitated. Strip 0 is hCG—P2 in 1× iso buffer. Note: The side products are likely caused by the formation and extension of primer dimers.

Figure 2. Sensitivity and selectivity of synthetic ZEBOV DNA plasmid in 15% human saliva (A) and in 10% human serum (B) using LAMP-to-hCG transduction. The hCG strip (inset) responses to different amounts of target (ZEBOV) templates and off-target (BRAF) templates are quantitated. Strip 1: negative control without templates; strip 2: 20 copies; strip 3: 200 copies; strip 4: 2 × 10^3 copies; strip 5: 2 × 10^4 copies; strip 6: 2 × 10^5 copies of ZEBOV templates; strip 7: side products; strip 8: quantitated. Strip 0 is hCG—P1 in 1× iso buffer. Note: The side products are likely caused by the formation and extension of primer dimers.

Figure 3. Distinguishing SNPs using strand-displacement (OSD) probes. A) hCG strip responses to both WT- and SNP-BRAF templates with either hCG—WT or hCG—SNP reporters. B) Quantitation of (A) using Image J software.

Figure 4. A) hCG strip responses to both WT and SNP-BRAF templates with hCG—WT and hCG—SNP reporters. B) Quantitation of (A) using Image J software.

Nucleic acid amplification assays often rely on the ability to discriminate SNPs in samples, in order to discern exactly which variant of a gene or organism is present. We have previously shown that so-called oligonucleotide-strand displacement (OSD) probes can be used for SNP detection, and have now adapted OSD probes to our pregnancy test kit transduction assay (Scheme 1B). The hybridization of the reporter to the correct target loop is initiated at the single-stranded toehold and then proceeds via branch migration, leading to dissociation of the shorter signal strand. The V600E mutation of the BRAF gene is a melanoma-associated biomarker and LAMP primers that incorporated either the wild-type (WT) BRAF sequence or the V600E BRAF (SNP-BRAF) T-to-A transversion mutation in the loop region were used to discriminate wild-type and mutant alleles. In parallel, hCG-labeled OSD reporters (hCG—WT and hCG—SNP) were designed that would be activated by the amplified WT- and SNP—BRAF loop sequences, respectively. LAMP-OSD-to-hCG strip transduction was carried out with 0 and 2 × 10^6 copies of the WT-BRAF gene and SNP-BRAF. When the hCG—WT reporter was introduced, no band appeared on the test line for the WT-BRAF LAMP amplicons, as expected (Figure 3). In contrast, when the hCG—SNP reporter specific for the V600E allele was used, the mutant gene could be detected.
To reconfigure strand-exchange reporters for “signal-on” detection with pregnancy test strips, these advances were abetted by protein-engineering efforts that yielded a single attachment point for oligonucleotide probes to hCG. By capturing hCG–DNA probes (Scheme 1A), as few as 20 copies of ZEBOV virus nucleic acid biomarkers could be detected after a 1.5 h isothermal amplification reaction and a YES-or-NO answer for the presence of the ZEBOV virus could be obtained in buffer (Figure 1A), 15% human saliva (Figure 2A), and 10% human serum (Figure 2B). Similarly, as few as 2 × 10^5 copies of a melanoma-relevant BRAF sequence could be detected using a pregnancy test kit (Figure 1B). This limit of detection is physiologically relevant, but higher than observed in our previous work with this biomarker (20 copies)[14] because we limited ourselves to a shorter, more clinically relevant LAMP reaction time. Finally, we have also demonstrated the storage of lyophilized hCG–DNA probes at room temperature for at least 90 days without losing activity.

The sensitivity of these assays comes primarily from the powerful signal-amplification properties of LAMP, while selectivity comes from both the LAMP primers and the novel strand-exchange reactions. Strand-exchange reactions can be engineered for specificity, and previous work has demonstrated that toehold design can significantly modulate the kinetics of strand displacement.[21,22] We have previously shown that even a single mismatch between a LAMP amplicon and a toehold sequence can greatly reduce strand displacement and squelch background.[14] We reconfigured hCG capture to be dependent upon the initiation of strand exchange (Scheme 1B), and wild-type and BRAF V600E could be detected by their respective, matched probes, but not by mismatched probes (Figure 3). The discrimination afforded by mismatches with the short toehold sequence may be further amplified because of competition between the OSD reporter and the Fc-loop for the F-loop (since the Fc-loop is complementary to the F-loop; Scheme 1B).[14]

To reconfigure strand-exchange reporters for “signal-on” detection with pregnancy test strips, we engineered a kinetically trapped three-way junction reporter (Scheme 1C), and assembled it on magnetic beads bearing a longer, toehold-containing strand (P7). Interaction of the F-loop with P7 and of the Fc-loop with P8 results in the release (rather than capture) of a short oligonucleotide–hCG conjugate. Using this signal-on strategy as few as 20 copies of the ZEBOV surrogate could be detected relative to negative controls, including in LAMP reactions conducted in 5% human serum (Figure 4). The signal-on strategy incorporated some of the design features used for allele discrimination, since toehold initiation must first occur for the overall strand-exchange reaction to proceed.

The advantage of using nucleic acid strand exchange in analytical methods is that it can be readily transferred between platforms. By focusing on molecular transduction rather than on the development of a wholly new diagnostic platform it proved possible to develop rapid tests that could potentially be directly used for public health monitoring.

Acknowledgements

This study was supported by the William and Melinda Gates Foundation (OPP1028808), the National Institutes of Health (U54EB015403), the National Security Science and Engineering Faculty Fellowship (FA9550-10-1-0169), the Welch Foundation (F-1654), and the Cancer Prevention Research Institute of Texas (RP140315).

Keywords: human chorionic gonadotropin · loop-mediated isothermal amplification (LAMP) · nucleic acid amplification · pregnancy test strips · SNAP protein


Received: September 17, 2016
Published online: ■■ ■■. ■■■■■
Alternative use for pregnancy test strips: By engineering a fusion reporter protein of human chorionic gonadotropin (hCG) and the SNAP protein the hCG–DNA conjugate was synthesized. In addition, isothermal amplification assays were coupled to strand-exchange nucleic acid transducers and thereby hCG signals were generated that can be directly read by off-the-shelf pregnancy test strips.