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Point Detection of Pathogens in Oral Samples

Abstract
We have outlined our progress with respect to developing a novel device for monitoring oral samples for bacterial and/or viral pathogens. The system is based on an existing device for measuring drugs of abuse in an oral sample. The sample is collected on an absorbent pad that delivers a metered dose to the cassette. The sample is then separated into 4 channels for the detection of antigen, RNA or DNA, and host antibodies to the pathogen. The detection system involves the Upconverting Phosphor Technology (UPT), whereby the captured pathogen analyte is detected by interrogation of the UPT particles with near-infrared light, and the emitted visible light is detected by the analyzer. Several of the steps in this process have already been worked out for viral and/or bacterial pathogens, and most of the remaining effort will be aimed at integrating these steps into a single microfluidic device while maintaining the current sensitivity.

Keywords
Microfluidics, particle technology, up-converting phosphor technology (UPT), bioassays, point-of-care diagnostics, lateral flow

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Point Detection of Pathogens in Oral Samples

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Introduction

We are developing a novel, oral-based system, designed for use in the diagnosis of multiple infectious diseases. Key to this project is a core technology that permits the simultaneous analysis of viral and/or bacterial antigens and nucleic acids, as well as antibodies to these pathogens. While the project is focusing on HIV, *B. cereus*, and *V. cholerae* for proof of principle, the platform will be applicable for the detection of many other infectious agents, including those associated with bioterrorism.

The detection of human pathogens with this system involves first obtaining a biologic specimen (blood, urine, saliva, oral mucosal transudate, nasal swab, etc.). Microfluidic devices will be used to propel and channel the sample, which then binds to a specific target zone for subsequent detection. Current methods to detect analytes in biological samples typically involve a reporter to identify the desired target (Kricka, 1999; Whitcombe *et al.*, 1998). The reporters may be enzymes/substrates, chemiluminescence, fluorescence, radiolabels, quantum dots, or visual determinations. Each of these has potential problems associated with the background signal that results in a decrease in the signal-to-noise ratio, resulting in low analytical sensitivity. This, in turn, complicates the investigator's ability to develop a multiplexed assay for detection of several analytes in a single test.

Our platform is based on a phosphor particle technology referred to as Up-converting Phosphor Technology (UPT). These phosphors absorb 2 or more photons at a low frequency and emit a signal at a higher frequency. Thus, UPT particles absorb infrared light and emit visible photons. Since this up-conversion relies on combinations of lanthanide-containing crystals that do not exist in biological materials, sensitivity of the UPT system is high, and the background is low (Zijlmans *et al.*, 1999; Hampl *et al.*, 2001). Assays and devices based on UPT have been developed to detect antigens and antibodies (Niedbala *et al.*, 2001), as well as nucleic acid sequences of pathogens (Corstjens *et al.*, 2001; Zuiderwijk *et al.*, 2003). UPT has also shown improved sensitivity in nucleic acid microarray applications (van de Rijke *et al.*, 2001).

The integrated system will collect and then introduce an oral sample into a cassette and process it through 4 pathways to detect pathogen-derived antigens, RNA, DNA, and host antibodies to that pathogen. This will provide detailed identification of viruses and/or bacteria and information on the host immunological responses to those organisms. The overall process design is diagrammed in Fig. 1. The detection with phosphorescent UPT particles is carried out in the UP*link* reader (Fig. 2). The UP*link* reader utilizes a low-power near-infrared laser that interrogates the lateral flow strips. Note that the UPT particles convert 2 or more photons of infrared light to higher-energy visible light.

In this paper, we describe our progress in: (1) collector studies, to identify the optimal collector for oral samples; (2) evaluation of UPT sensitivity, as compared with a visual immunogold technique; (3) microfluidic processing, development of novel flow and PCR devices compatible with the design pathway; and (4) multiplex detection formats.

Collector Studies

To develop a UPT-based system using oral samples to detect multiple pathogens, we originally assessed 9 commercially available oral collectors (Fig. 3A). Four of these collectors were evaluated (see red box in Fig. 3A) in terms of their ability to pick up and deliver fluid (saliva or buffer), protein (amylase), and *B. cereus* (Holm-Hansen *et al.*, 2004). In addition, we demonstrated that samples of bacteria collected with all of the collectors were compatible with subsequent DNA PCR, an important consideration for the anticipated use of this technology. Five collectors were excluded from the final study based on esthetic qualities, low sample volume transferred, or incompatibility with the system being developed. The remaining four collectors were evaluated for ability to collect and deliver fluid, protein, bacteria, and DNA. While each of the collectors demonstrated specific advantages, we selected the UP*link* collector as most suitable for this project. The UP*link* collector is designed to deliver a metered fluid sample directly into the lateral flow (LF) cassette (Fig. 3B). Within the cassette, the fluid sample received on the sample application pad flows past the UPT particles containing a biologic conjugate (antigen, antibody, or short nucleic acid sequence), which then flows past the target zones containing the specific capture reagent (Fig. 3C).

Using the schematic shown in Fig. 1 as a guide, we have begun to construct and evaluate each of the individual pathways. After selection of the collector, studies focused on incorporating PCR into the process, since this analysis is the most complex aspect of the microfluidic platform (Kricka and Wilding, 2003).

Evaluation of UPT Sensitivity

To determine the sensitivity of UPT-LF formats, we compared the detection of DNA PCR products with that in an immunogold LF system (Roche Diagnostics B.V., Almere, The Netherlands). Target DNA molecules were prepared by PCR amplification of a specific nucleic acid fragment. Primers with digoxigenin (Dig) or Biotin (Bio) hapten were applied to provide each target DNA molecule with 2 different labels. In the LF assay, the Bio hapten on the target DNA product was captured by avidin or streptavidin test

Key Words

Microfluidics, particle technology, up-converting phosphor technology (UPT), bioassays, point-of-care diagnostics, lateral flow.

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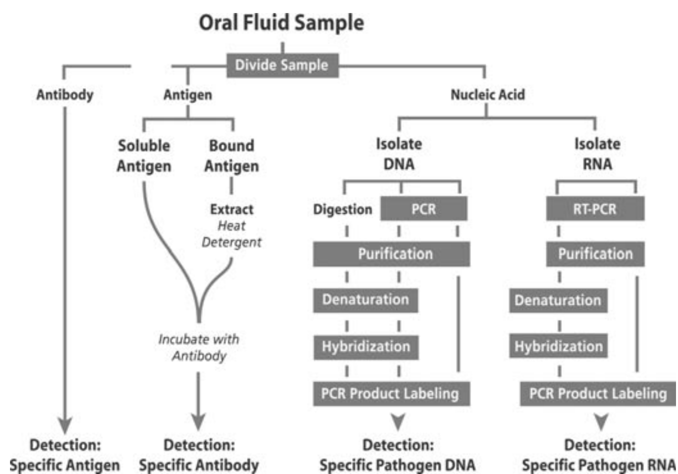


Fig. 1 — Schematic of overall sample-processing paths. An oral sample enters the system and is divided into 4 aliquots. For antibody detection, the sample binds to a specific antigen and is detected by UPT conjugated with antigen. Soluble antigens can be detected directly, while bound antigen will require extraction with heat or detergent, for example. In this path, antigen binds to a specific capture antibody and is detected by UPT conjugated with antibody. For DNA and RNA, it will be necessary to purify the sample partially and amplify it by PCR or RT PCR prior to UPT detection.

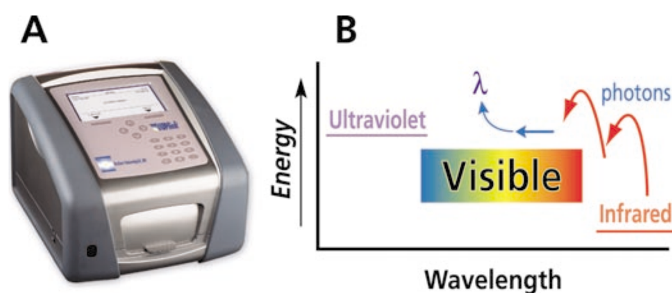


Fig. 2 — The UP*link* reader and IR up-conversion. (A) Existing UP*link* analyzer. (B) Schematic of Up-conversion process.

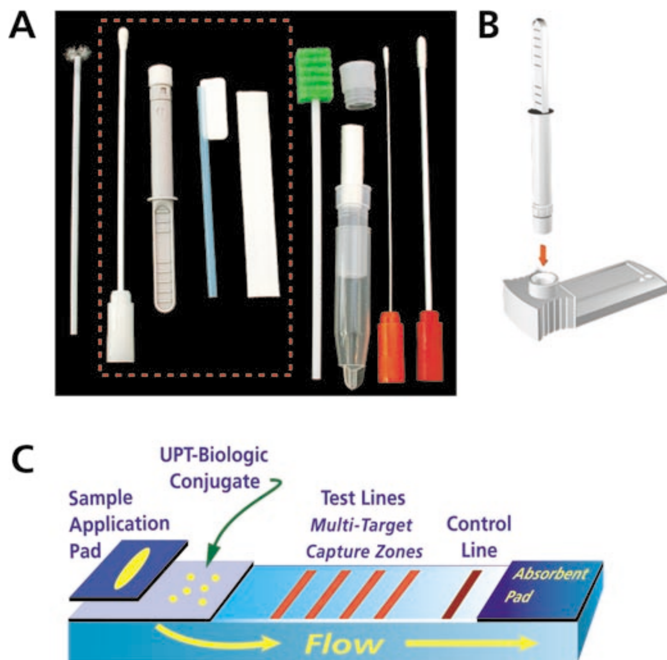


Fig. 3 — Commercially available collectors evaluated. (A) Collectors shown were obtained from the following sources: CytoBrush (Cooper Companies, Inc., Medscand Medical AB, Malmö, Sweden), BBL white cap (Becton Dickinson and Co., Sparks, MD, USA), UP*link* collector (OraSure Technologies Inc., Bethlehem, PA, USA), OraSure HIV-1 Oral Fluid Specimen Device (OraSure Technologies, Bethlehem, PA, USA), TRANSORB wicks (Filtrona Richmond Inc, Colonial Heights, VA, USA), Toothette-Plus Swabs (Sage Products Inc., Crystal Lake, IL, USA), BBL Culture Swab, orange cap (Becton Dickinson and Co.), and BBL red cap [EZ] (Becton Dickinson and Co.). The red-dashed box around four of the collectors indicates those chosen for detailed comparison. (B) The Orasure UP*link* collector and analysis cassette and (C) an annotated schematic of the lateral flow nitrocellulose strip in the UP*link* cassette.

lines on the lateral flow strips, whereas antibodies on the UPT and gold reporter particles specifically bound the Dig hapten label.

A dilution series of Dig-Bio tagged target DNA was analyzed by lateral flow. We initiated the assay by adding target DNA dilutions to the sample application pad, followed by a buffer containing the UPT conjugate. The results were compared with those achieved with commercial DNA detection test strips (Roche Inc.) utilizing anti-Dig gold conjugate for staining. With the latter test, a minimum of 1 ng of DNA was detectable (Fig. 4, panel A). Since the sample volume was 5 μL for this specific assay, a PCR yield of 0.2 ng/μL is required to get a positive signal; this is equivalent to a minimum target DNA concentration of 600 fM. By comparison, when the UPT detection system was applied (see Fig. 4B), ~ 10 pg was detectable, thus reducing the minimum target DNA to only 6 fM.

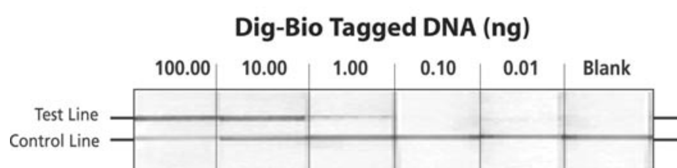
In the above-described format, sensitivity of detection with UPT was improved by a factor > 100 compared with LF-gold. Further improvement of sensitivity was observed when Dig-Bio tagged target DNA was pre-incubated with the UPT-conjugate prior to lateral flow. UPT-LF detection of ~ 10^6 non-amplified nucleic acid targets from *Streptococcus pneumoniae* has been demonstrated, utilizing hybridization with Dig and Bio tagged probes (Zuiderwijk *et al.*, 2003).

Microfluidic Processing

For nucleic acid analysis, we explored several PCR and RT-PCR design types. The first system, referred to as Self-Actuated Reactor (Chen *et al.*, 2004), relies on the temperature variations in PCR (94, 55, 72°C) to produce density variations in the fluid, which in turn produces buoyancy forces and a continuous flow circulation in a sealed system. This device was tested with *B. cereus* genomic DNA and found to be as effective as the traditional bench-top PCR unit in producing the correct size amplicon (305 bp).

A second system utilizes a pneumatic oscillatory device to move the reactants among the 3 heating zones (Fig. 5). This system has also been used successfully on genomic *B. cereus* DNA. The data shown in Fig. 5B present the results from a 60-minute PCR reaction. By optimization of each step of the reaction, we have been able to reduce this time to 28 min, and have set a goal of 10-15 min for the nucleic acid amplification steps. Note that the PCR product from the pneumatic PCR is equivalent to that obtained with a standard bench-top PCR. Furthermore, the amount of amplicon produced is influenced by both cycle number and taq concentration.

Since the LF-UPT detection/UP*link* system has a high sensitivity, and since the analyzer and software already exist, our plan is to modify the current cassette so that antigen, antibodies, RNA, and DNA can be detected simultaneously. Currently, it is possible to detect each of these analytes individually, with nucleic acid amplification carried out with the use of a bench-top PCR unit. We now plan to build an integrated microfluidic system, including PCR amplification, to accomplish a multiplexed detection format.



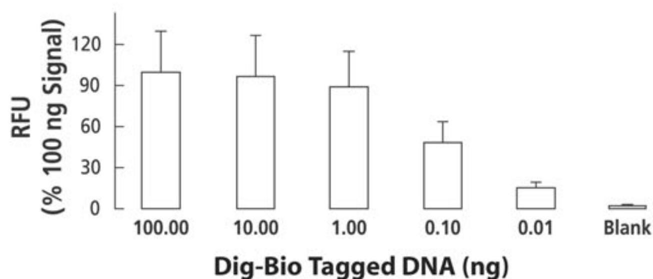
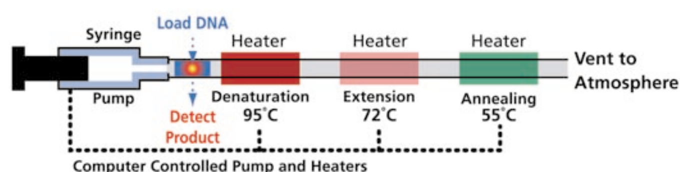


Fig. 4 — Comparison of immunogold detection with UPT detection. **(A)** The results obtained with LF-gold (strips containing an integrated gold conjugate pad). The upper line is the test line (streptavidin), and the lower line is the flow control line. **(B)** The results obtained with LF-UPT. The lateral flow strips used for LF-UPT contained an avidin test line and an anti-mouse antibody control line, as described earlier (Corstjens *et al.*, 2001) on high-flow nitrocellulose (SRHF04000; Millipore Inc.). LF-UPT analysis was performed with 100 ng of anti-Dig UPT conjugate. UPT signals represent visible emission after IR excitation of the test line; signals are normalized to the signal obtained with 100 ng Dig-Bio tagged DNA.



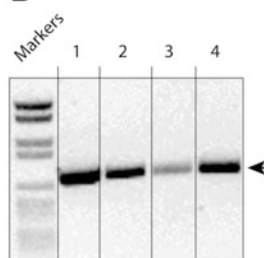
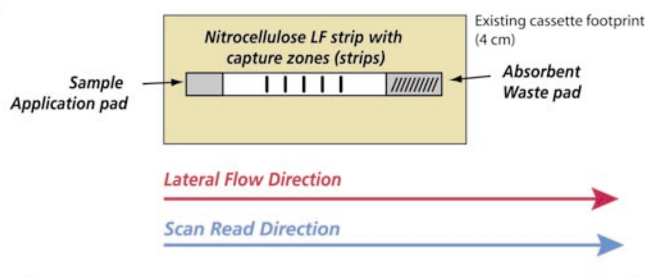


Fig. 5 — Pneumatic microfluidic oscillatory PCR reactor. **(A)** A schematic of the PCR device. **(B)** Ethidium-bromide-stained agarose gel of PCR products [Lane 1, control (benchtop thermocycler); lane 2, pneumatic, 35 cycles; lane 3, pneumatic, 25 cycles; lane 4, pneumatic, 25 cycles, 2X taq]. Arrow indicates the size of the anticipated PCR product of 305 bp.



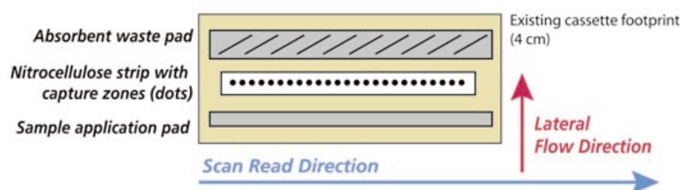


Fig. 6 — Multiple capture lines on conventional lateral flow strips and Trans-Dot (TD) strips. **(A)** Diagram of a conventional, parallel lateral flow format. **(B)** Schematic of a Trans-Dot (TD) lateral flow strip.

Multiplex Detection Formats

Multiple target capture zones: Trans-Dot strips

Improved detection sensitivity in rapid lateral flow systems, through incorporation of UPT, facilitates increased assay robustness, and capture of multiple targets utilizing a linear array of various trapping molecules appears feasible.

Multiple target-capture molecules can be aligned perpendicular or parallel to the flow (Fig. 6). Perpendicular placement of capture zones is preferred, since it eliminates differences in signal intensity, since the distance from the sample pad to all capture zones is constant. Moreover, perpendicular placement eliminates the requirement of the sample to pass through alternate capture zones. Potential interference of preceding target capture zones can thus be ignored in assay development.

Lateral flow strips with perpendicularly placed capture zones (Trans-Dot strips) were developed to fit in the UP*link* cassette so that scanning was possible in the existing UP*link* reader. A prototype of the TD-cassette is shown in Fig. 7. Modifications were accomplished such that the existing scanning/read window remained in place.

Nanoliter capture spots were prepared with the use of a BioDot apparatus (BioDot Inc., Irvine, CA, USA). For initial experiments, 4-cm-wide strips were used with 2-mm anti-Bio antibody dots separated by 1-mm BSA dots. The BSA dots were added to help create a continuous flow pattern by generating an uninterrupted protein barrier. A serial dilution of Dig-Bio DNA (a 500-bp PCR fragment from *Vibrio cholerae*) was analyzed following pre-incubation of the DNA with the UPT-conjugate. The lowest amount of DNA tested (1 ng) was detected with a background-to-signal ratio of approximately 10 (Fig. 8), signals obtained with 10 ng of DNA or higher saturated and clipped at 60,000 RFU. This configuration can accommodate up to 400 μL of sample in a total assay volume of 500 μL, so this particular assay yields a sensitivity of 6 pM. Note that the signal response in all 8 capture dots is highly reproducible. In comparison with conventional lateral flow strips, TD strips allow a larger sample volume to be used. The expected detection sensitivity with TD is in the high-fM range. Sensitive detection of multiple antigens, antibodies, or abundant (non-amplified) nucleic acids in a single sample is feasible with this system.

The consecutive-flow format

UPT labels are valuable for the simultaneous detection of multiple analytes in a single sample. Initial coupling, with a Universal Linkage System (ULS®; KREATECH Biotechnology B.V., Amsterdam, The Netherlands), of the entire population of macromolecules present in a sample allows for the use of generic UPT labels. However, this requires a capture system that enriches for the analytes of interest prior to binding of the generic UPT reporter. To provide such enrichment in a lateral flow system, the ULS tagged sample was 'flowed over' the lateral flow LF strip prior to the UPT reporter flow. The ULS-tagged targets of interest bind to their specific capture zones on the LF strip, and the unbound analytes are removed by washing. Finally, a subsequent flow with the generic UPT

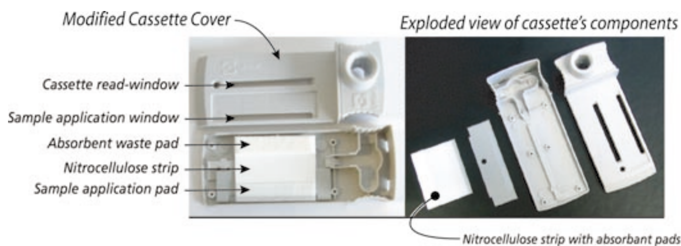


Fig. 7 — Cassette modified for transverse UP*link* UPT scanning. Note that a new window, the sample application window, has been added, and the 'cassette read' window remains intact.

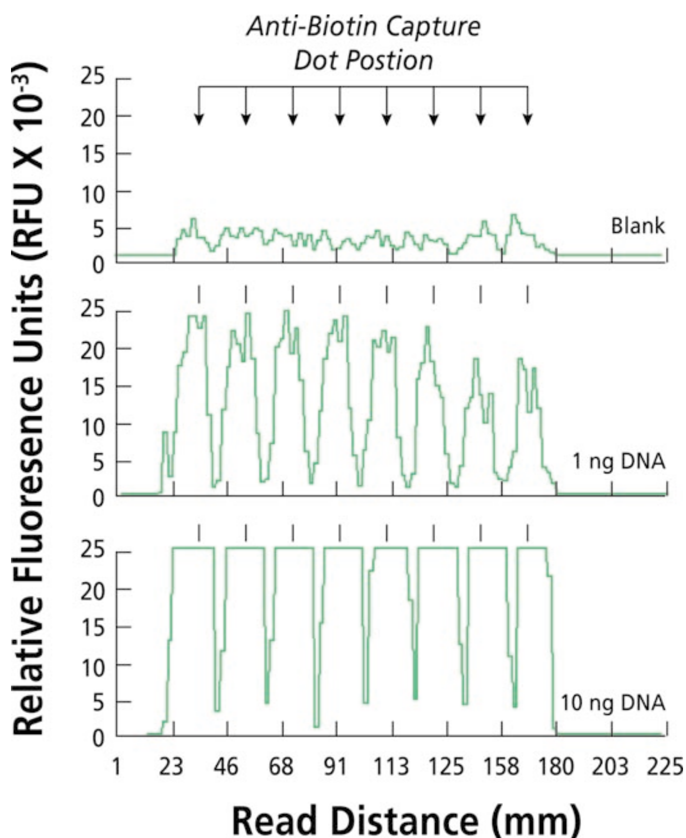


Fig. 8 — Analysis with BioDot Trans-Dot strips. The **top panel** represents the blank (no DNA), the **middle panel** shows the signal obtained with 1 ng DNA, and the **bottom panel** that with 10 ng DNA. Note that the highest sensitive scale was plotted for all 3 panels, to allow sensitivity to be compared with the blank.

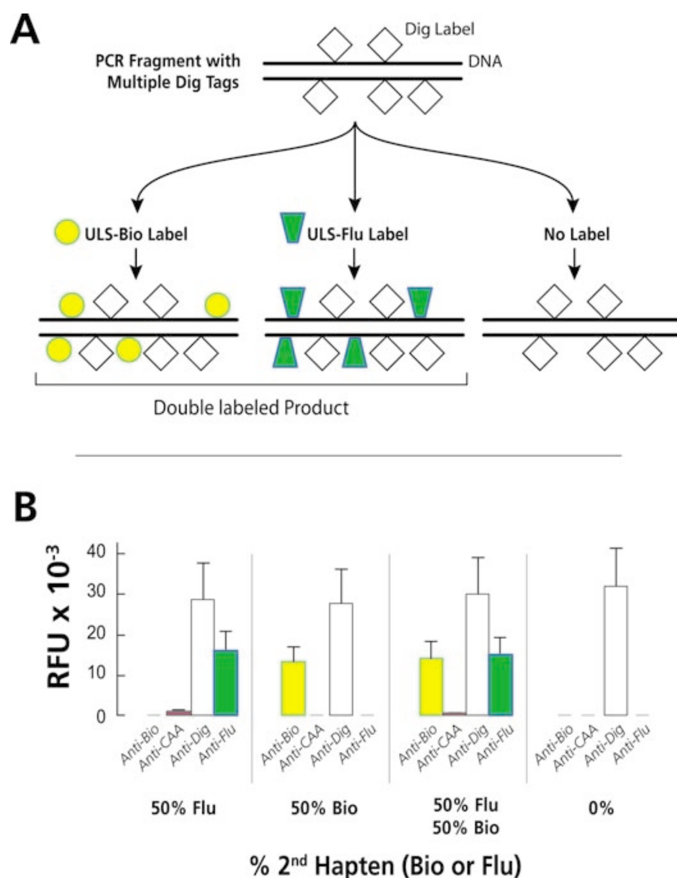


Fig. 9 — Universal labeling and consecutive flow with a generic UPT reporter. **(A)** Diagram of Universal Labeling System (ULS). **(B)** The total amount of a 500-bp DNA fragment from *Vibrio cholerae* in each sample was kept constant at 3 ng, so that each sample contained the same amount of digoxigenin hapten. The 50% Flu sample contained 1.5 ng of Flu-Dig and 1.5 ng of Dig-tagged DNA, and the 0% Flu sample contained 3 ng of Dig-tagged DNA only. DNA mixtures were added to 200 μL of running buffer and flowed over a 2.5-cm-wide TD strip spotted with 25 ng of anti-Bio, anti-CAA, anti-Dig, and anti-Flu antibody. As soon as the sample was adsorbed by the sample pad, an additional 100 μL of buffer was applied. Finally, 200 μL of buffer containing 1 μg of UPT particles was applied to the TD strips, and strips were allowed to air-dry for 5 min before IR scanning occurred.

reporter label is performed, allowing the reporter to bind to the Dig tag at locations on the strip where specific binding of the ULS-tagged target has occurred. This flow format is referred to as the 'consecutive-flow' format.

The feasibility of the above-described consecutive flow was tested with a 500-bp *Vibrio cholerae* DNA fragment provided with multiple Dig hapten by PCR by incorporation of digoxigenin-11-dUTP nucleotides (Fig. 9A). Subsequent ULS labeling was used to provide part of the Dig-tagged DNA with either fluorescein (Flu) or biotin (Bio). Various combinations of Dig, Dig-Bio, and Dig-Flu DNA were combined and tested with consecutive flow and an $UPT^{M\alpha Dig}$-conjugate. The capture of the various hapten (Dig, Flu, and Bio)-tagged DNA fragments did not show any cross-interference (Fig. 9B). In the assay, the amount of Dig hapten was kept constant, while the amounts of Bio and Flu hapten were varied. Signals detected were directly related to the total amount of a specific hapten present in the sample. The signal was not influenced by the number of different haptens present in the sample. This experiment demonstrates the feasibility of the combination of TD strips, consecutive flow, and ULS and a generic UPT label. The detection of multiple antibodies and antigens can be envisaged with this system.

Compared with gold, the application of UPT in lateral flow formats leads to 100-fold-improved sensitivity. It has been demonstrated that, in fully optimized UPT-LF systems, the current limit of detection is ~ 10^6 target molecules. (With a 100-μL sample volume, this would be the equivalent of a sample with a target concentration in the range of 1 fM.) This enhancement in sensitivity allows for expansion of the assay's complexity, *i.e.*, testing for the presence of more than one target in a single sample. In this respect, the consecutive-flow format, combined with trans-dot strips, may be ideal for multiplex assays. Moreover, the combination of TD with target labeling and consecutive flow allows for the convenient usage of a generic UPT label.

Summary

We have outlined our progress with respect to developing a

novel device for monitoring oral samples for bacterial and/or viral pathogens. The system is based on an existing device for measuring drugs of abuse in an oral sample. The sample is collected on an absorbent pad that delivers a metered dose to the cassette. The sample is then separated into 4 channels for the detection of antigen, RNA or DNA, and host antibodies to the pathogen. The detection system involves the Up-converting Phosphor Technology (UPT), whereby the captured pathogen analyte is detected by interrogation of the UPT particles with near-infrared light, and the emitted visible light is detected by the analyzer. Several of the steps in this process have already been worked out for viral and/or bacterial pathogens, and most of the remaining effort will be aimed at integrating these steps into a single microfluidic device while maintaining the current sensitivity.

Acknowledgments

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