Rapid Assay Format for Multiplex Detection of Humoral Immune Responses to Infectious Disease Pathogens (HIV, HCV, and TB)

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ABSTRACT: A novel assay is described for multiplex detection of antibodies against different pathogens from a single sample. The assay employs a modified lateral flow format (consecutive flow, CF) together with a sensitive reporter particle technology (up-converting phosphor technology, UPT) that allows for fully instrumented assay analysis. Lateral flow (LF) strips developed for the detection of human antibodies against human immunodeficiency virus type-1 and -2 (HIV-1 and -2) with additional capture zones to detect antibodies against Myobacterium tuberculosis (TB) and hepatitis C Virus (HCV) provided the strips to test multiplexing. Data are presented that show the performance of the TB and HCV test, as well as two multiplex assays, TB with HIV and HCV with HIV. The TB/HCV assays demonstrate excellent detection capability, and HIV multiplexing does not affect the qualitative test result. The bench-top CF format was converted to a microfluidic platform and a first prototype semiautomated chip capable of performing CF is presented here.

KEYWORDS: infectious disease; multiplex assay; up-converting phosphor; lateral flow; TB; HCV; HIV

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INTRODUCTION

The presented antibody test platform allows convenient, rapid, sensitive, and cost-effective detection of infectious disease pathogens. It was initially developed for the detection of antibodies to HIV. In this report we explore the potential of the platform for multiplex detection of antibodies associated with other infectious diseases, for example, simultaneous detection of HIV, TB, and HCV. TB often causes opportunistic infection in HIV/AIDS patients, and HCV has a high prevalence in HIV-infected individuals and is suspected to co-infect with HIV. The antibody test platform was designed to become part of a modular microfluidic device, a point-of-care (POC) device to analyze oral fluid for simultaneous detection of pathogenic antigens, nucleic acids, and the host antibodies to the pathogen.

The antibody assay uses a modified LF immunochromatography format with three consecutive flow (CF) steps: (1) the first flow with diluted specimen (e.g., plasma, oral fluid, or urine); (2) the second flow with wash buffer; and (3) the third flow with protein A–coated reporter particles. The multiple flow system (CF) accommodates a 10-fold higher sample volume compared to a single flow format, and demonstrates a better signal-to-noise value. Previously described up-converting phosphor technology (UPT) reporter particles are applied for ultrasensitive and instrumented assay analysis. These UPT reporters generate a visible light emission signal upon excitation with low-energy infrared (IR) light. Interrogation of LF strips exposed to UPT reporters can be performed with a portable UPT reader (UPlink). In the UPlink system LF strips are integrated in disposable plastic cassettes. We developed and constructed a prototype semiautomatic microfluidic module to perform CF that fits into existing UPlink-compatible cassettes.

RESULTS

Consecutive Flow

Figure 1 shows an illustration of UPT–CF designed for detection of human antibodies against HIV. Antibodies to HIV-1 and -2 are captured at the test line which consists of HIV-specific antigens and the remaining human IgGs bind downstream to an anti-human IgG flow-control line. Nitrocellulose sheets for CF with proprietary HIV and HCV test lines were provided by OraSure Technologies. A third test line with a proprietary TB antigen mixture (Courtesy of H.J. Houthoff and G.J. van Dam) was added to the CF strips in-house using a Camag Linomat IVstriper. Nitrocellulose sheets with only a TB-specific test line were prepared in-house in a format similar to the sheets with the HIV-specific test line. Nitrocellulose sheets were assembled into LF strips as described earlier. The assays described here used plasma, but can be easily
FIGURE 1. Consecutive flow format and antibody multiplex strips. (A) Consecutive flow for plasma samples comprises three successive flow steps: (1) diluted specimen; (2) wash buffer; and (3) protein A–coated UPT reporter particles. Bold capture lines (T and FC) indicate the deposition of the UPT particles that excite green light upon excitation with infrared light. (B) Schematic illustration of an antibody multiplex strip for the detection of antibodies against TB, HCV, and HIV.
modified to allow testing with oral fluid or urine. For bench-top assays, plasma samples are diluted 100-fold in assay buffer (100 mM Hepes, pH 7.2, 270 mM NaCl, 0.5 % Tween-20, 1% w/v BSA). The dilution factor will vary, depending on the type of specimen. After dilution, 10 μL is mixed in a microtiter plate well containing 40 μL assay buffer and flow initiated by inserting the LF strips into the well, followed by a 20 μL assay buffer wash immediately after the sample has migrated into the LF strip. These two flow steps take approximately 2 min, after which LF strips are added to a new well containing 70 μL of assay buffer with 100 ng UPTprotA conjugate. An illustration with a schematic presentation of a microfluidic device capable of mimicking the above bench-top CF format is shown in Figure 2. The microfluidic device differs from the bench-top assay in that the sample input is approximately 2 μL of either diluted sample or undiluted sample. Oral fluid specimens do not require dilution, whereas plasma needs to be diluted 10- or 100-fold prior to applying the sample to the loading well.

**FIGURE 2.** Semiautomatic prototype microfluidic device for consecutive flow. Pneumatic pressure is used to prefill the three buffer metering chambers. The specimen (around 5 μL) is applied to sample loading well, and cooling of the pressure chamber creates a void that pulls an approximately 2-μL specimen into the capillary system. Pneumatic pressure and ice valves operated by thermoelectric modules control the liquid flow.
**HIV/TB Multiplexing**

A collection of 300 banked plasma specimens (provided by H.J. Houthoff and G.J. van Dam) were analyzed with UPT–CF. The specimens consisted of 100 healthy control samples (HIV–/TB–), 100 samples from HIV-negative but TB-infected patients (HIV–/TB+), and 100 samples from patients infected with HIV and TB (HIV+/TB+). The UPT assay threshold for plasma samples as determined from an earlier study was verified by analyzing samples on LF strips prepared with an HIV-specific test line only. In this experiment, samples generating a ratio signal (test signal divided by flow-control signal) >0.04 were considered antibody-reactive. A ratio value rather than an actual UPT signal value (measured in relative fluorescent units, RFU) is preferred as this provides a convenient method to normalize test results; however, normalization is not a requirement. TB infection status was then tested on LF strips carrying a TB-specific test line only. The resulting clinical parameters regarding TB testing are presented in Table 1 together with lab-based enzyme-linked immunosorbent assay (ELISA) results that were provided with the specimens. From these results we concluded that the accuracy of the rapid UPT assay is superior to the lab-based ELISA assay; it is important to note that both assays used the same antigen mixture to capture TB-specific antibodies, so that the increased performance is related to the difference in assay platform (ELISA vs. LF) as well as the difference in reporter technology (fluorescence vs. UPT).

Compared with testing for other infectious diseases, the actual specificity and sensitivity value for TB is relatively low, which is a known problem. Attempts to develop a low-complexity, rapid, immunologic-based TB assay with sufficient accuracy have failed so far. In developing countries TB diagnosis is especially problematic because children and HIV-compromised patients are low responders, which increases the number of false negatives.

**TABLE 1. UPT consecutive flow analysis of plasma specimen from 200 TB-diagnosed patients (50% being HIV compromised) and 100 healthy controls**

<table>
<thead>
<tr>
<th>TB/HIV Statusa,b</th>
<th>Assay</th>
<th>Mixed (n = 300)</th>
<th>Without HIV+/TB+ (n = 200)</th>
<th>Without HIV–/TB+ (n = 200)</th>
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</thead>
<tbody>
<tr>
<td>HIV–/TB– (n = 100)</td>
<td>ELISAb</td>
<td>96.0</td>
<td>95.0</td>
<td>96.0</td>
</tr>
<tr>
<td>HIV–/TB+ (n = 100)</td>
<td>UPT</td>
<td>96.0</td>
<td>95.0</td>
<td>96.0</td>
</tr>
<tr>
<td>HIV+/TB+ (n = 100)</td>
<td>ELISAb</td>
<td>96.0</td>
<td>95.0</td>
<td>96.0</td>
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</tbody>
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aPlasma specimens of 200 TB-diagnosed patients (TB+) and 100 healthy controls (HIV–/TB–) were analyzed. Half (n = 100) of the TB-diagnosed patients were also diagnosed as HIV+.

bPlasma specimens and corresponding ELISA data were provided by H.J. Houthoff and G.J. van Dam.
indicates that the UPT assay sensitivity improves from 62.5% to 74.0% when the HIV-compromised patient group (HIV+/TB+ samples) is omitted. This is also evident when examining the ELISA data.

Analysis of selected samples on multiplex LF strips with TB and HIV test lines did not affect the qualitative result of the assay (results not shown). A detailed study is ongoing to examine the potential effect on signal value of “preceding capture line interference,” including differences in the location/distance of the capture line from the sample application pad.

**HIV/HCV Multiplexing**

The UPT–CF format was also used for the detection of HCV infection. Analysis of three seroconversion panels demonstrated excellent performance of the UPT assay compared to an EIA (TABLE 2), which demonstrates the applicability as a model in developing UPT–CF multiplex assays. To further explore the extent of potential “preceding capture line interference,” a “dilution checkerboard matrix” of a high-reactive HCV panel member mixed with a high-reactive HIV plasma sample was prepared in normal human plasma (NHP) and analyzed on multiplex LF strips. Each multiplex LF strip produced an HIV as well as HCV test signal, and a flow-control signal. HIV and HCV ratio signals were calculated by dividing their individual test signal by the joint flow-control signal. In Figure 3 the ratio value is indicated on the y axis of three-dimensional histograms; panel A shows the results for HIV and panel B shows the results for HCV. In both histograms the x- and z axis represents the

<table>
<thead>
<tr>
<th></th>
<th>BBI Panel PHV905&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BBI Panel PHV907&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BBI Panel PHV914&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>Days&lt;sup&gt;b&lt;/sup&gt;</td>
<td>EIA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>UPT&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Days&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.02</td>
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<td><strong>2.00</strong></td>
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<sup>a</sup>Detailed panel information at www.seracare.com/bbidx/hcv_panels.htm.

<sup>b</sup>Days after first bleed.

<sup>c</sup>BBI-provided data; test values ≥ 1 were considered positive (indicated in bold).

<sup>d</sup>UPT ratio values above > 0.04 were considered positive (indicated in bold).
FIGURE 3. Result of a multiplex analysis; the simultaneous detection of antibodies against HIV and HCV in single plasma specimens. A dilution series of plasma specimens from an HIV-compromised patient and an HCV-compromised patient were mixed in equal amounts. In the histograms “0” indicates the highest amount of HIV and/or HCV-compromised plasma specimen; the indicated dilutions were made in NHP. NHP is also used as the (no signal) control sample. The dilution of HIV plasma is indicated on the x axis, the dilution of HCV plasma (z axis) (A) the HIV ratio signal, (B) the HCV ratio signal. All data points are the average value of three individual experiments.

test matrix with the HIV dilution on the x axis and the HCV dilution on the z axis. The experiment was performed in triplicate and the average signal is presented in the histograms. For purpose of comparison the maximum ratio value was normalized to 1.

The actual ratio value as determined for undiluted HIV and HCV samples was 0.96 and 6.0, respectively (in FIGURE 3 these values were normalized to 1). In patients infected with both viruses, HIV and HCV ratio signals expectedly are different because HIV and HCV test lines use different capture antigens to detect their respective antibodies; the amount of antibodies specific against HIV and HCV is variable, as is the binding affinity of the applied antigen–antibody pairs. In the experiments performed here the HCV test line was closer to the sample application pad than the HIV test line (Fig. 1). As a consequence, the HCV test line signals are higher in comparison to assays where the same HCV test line is localized at the (further downstream) position of the HIV test line. In theory this implies an assay cut-off threshold value that is dependent on the distance of the test line from the sample application pad. For the experiment described here, the actual test line ratio signal obtained on multiplex LF strips with NHP (x5, z5 in Fig. 3) generated approximately the same value for HCV and HIV (respectively 0.032 and 0.027 [n = 3]) and the maintained cut-off threshold value was > 0.04.
DISCUSSION

We describe a modified lateral flow format (CF) for antibody detection and explore its potential for multiplexing. The CF format was originally developed to detect human antibodies against HIV-1 and -2. Multiplexing is achieved by providing the HIV-specific LF strips with additional capture lines specific for antibodies against other pathogens. Similar to the HIV-specific test line, the additional capture lines comprise pathogen-specific antigens. The different test lines are placed transversely across the LF strip such that the tested specimen will sequentially pass individual test lines. Antibodies present in the specimen can bind to these antigens. This procedure demands careful development and optimization of the various capture lines in order to avoid nonspecific binding and to prevent undesired cross-reactivity of the antibodies with preceding test lines. Furthermore, when applying an antibody-generic reporter, a reporter flow completely disconnected from the antibody flow is desired. CF applies an initial flow of specimen, followed by a wash step and a final flow with antibody-generic UPT reporter particles. The initial flow allows a free flow of the antibodies present in the specimen, so that the antibodies against various pathogens can enrich in the spatially separated capture zones. A wash step then removes non-specific-bound antibodies from the LF strip, and in the final flow UPT<sub>protA</sub> reporters bind to the antibodies in the pathogen-specific capture zones.

The results presented in this article indicate that multiple antibody test lines are feasible. Multiplex analysis of combined specimens with high loads of antibodies against HIV and HCV did not show relevant interference. Further studies are necessary to evaluate the maximum number of test lines that can be applied to a LF strip without disturbing the UPT reporter particle flow. In this context the effect of high signals in the test lines closest to the sample pad needs to be carefully analyzed. But as most specimens will only show antibody reactivity for a limited number of pathogens, disturbance of the reporter flow to further downstream test lines as a consequence of multiple coinciding high test signals may only be a theoretical problem.

In earlier studies using LF strips with a single test line and a flow-control line, ratio calculations of the test (T) divided by the flow control (FC) were applied. This normalization allows for interassay comparison of results obtained with different LF strips. Increasing the number of test lines will probably affect the normalization algorithm. When the number of test lines is increased, differences in distance and signal may become more evident. In the multiplex assays presented here for TB and HCV these effects were minor and did not affect the outcome of the multiplex tests. Note also that qualitative assays do not necessarily demand a ratio calculation (see, e.g., Mokkapati <i>et al.</i>).^7^ The CF format described here allows multiplex detection of various antibodies from a single specimen with an antibody-generic UPT reporter. As such,
the CF format suits its objective and was therefore selected as the antibody test module in a (modular) microfluidic device that permits simultaneous detection of viral and bacterial antigens, nucleic acids, and antibodies to these pathogens. The bench-top CF format was therefore converted to a microfluidic module and a first semiautomated prototype is presented.

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REFERENCES