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Flow-through, viral co-infection assay for resource-limited settings

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ABSTRACT

Here we present a new and rapid immunofiltration assay for simultaneous detection of HIV p24 and hepatitis B virus antigens. The assay platform is composed of a 13 mm nitrocellulose filter spotted with capturing bioprobes and inserted in a Swinnex® syringe filter holder. Samples and reagents are flown through the nitrocellulose filter by manual pressure on the syringe. A colorimetric detection allows for naked eye results interpretation. The assay provides sensitivity in the picomolar range in just 5 min, even using low volumes of sample in complex matrix. Probe deposition by spotting allows for flexible combinations of different capturing agents and multiple diagnoses; furthermore, the very simple and inexpensive set-up makes the syringe-based immunoassay on paper microarray a suitable diagnostic system for resource-limited settings.

1. Introduction

Conventional and standard methods for pathogen detection include cell culture, PCR and enzyme immunoassays, which are often laborious, require well-equipped dedicated laboratories and take from several hours to several days to perform. Conversely, increased access to diagnostic products requires the development of tests that can be used in remote areas (with no or minimal laboratory infrastructures) by people with little or no laboratory training and that meet the ASSURED criteria [1]. Ideally, for these applications, the detection platform should be simple to use and interpret, stable under a wide range of operating conditions (such as temperature and humidity), portable, disposable and able to perform multiplex tests. Many attempts have been focused on the development of tests based on microfluidic immunoassays targeted at low resource settings (LRSs) [2–4]. However, only a few are complete sample-to-result systems capable of testing blood samples [5–8]. Lateral flow tests (LFTs) may be seen as the gold-standard microfluidic platform in terms of cost, handling simplicity, robustness, market presence and number of lab-on-a-chip applications [9]. The amount of sample and the reagent consumption are moderate, and the concept is mainly used for qualitative and semi-quantitative assays, intended for untrained users. However, the simplicity of LFT is also its main drawback. Assay protocols within capillary driven, flow-through cellulose strips follow a fixed process scheme with limited number of unit operations. Moreover, the highly precise liquid handling and metering is extremely challenging. Other severe drawbacks of LFTs lie in the fact that multiple screening applications, though possible, are difficult to implement.

In LFTs, samples flow in a horizontal direction through a nitrocellulose (NC) membrane towards the adsorbent pad. The horizontal flow causes three major limitations [9,10]: assay time (determined by NC flow rate), "hook effect" (interference due to excess of antigen) and line interference, which makes it difficult to use multiple lines due to the front-line reaction interfering with the behind-line reaction. In immunofiltration assays, the sample and reagents are filtered sequentially through a porous membrane containing spots or lines of capturing antibodies. Succesful examples of immunofiltration assays for detection of small molecules were demonstrated for methamphetamine [11] and the herbicide simazine [12] using water-soluble polyelectrolytes as carriers for the assay reactants. Though highly sensitive, the proposed assays detect only single analytes.

Multiplexed flow-through immunoassays were developed for IgM and antigens specific to *Salmonella typhi* using a 96 well plate connected to a vacuum manifold [13] and a microfluidic cartridge driven by a pneumatic system [14]. Recently, a vertical flow immunoassay was introduced [9] simply by vertically stacking all the components of an LFT, resulting in the detection of C-reactive protein (CRP) concentrations from 0.01 to 10 μg/mL in 2 min. An immunoflow protein platform that actively pumps the sample through an NC membrane was developed, demonstrating detection by fluorescence of 1 pM CRP. Comparing the flow assay to protein microarrays with

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conventional incubation by shaking method the rate of binding is increased by a factor of 10, allowing reduction of assay time without compromising sensitivity. However, all of the above cited works use external actuators like vacuum manifolds or pumps to flow liquids through the membranes.

Here we present a new and rapid immunofiltration assay, suitable for LRSs, for simultaneous detection of HIV p24 and hepatitis B virus (HBV) antigens with sensitivity in the picomolar range. The assay platform is composed of a 13 mm nitrocellulose filter spotted with capturing bioprobes and inserted into a Swinnex® syringe filter holder. Samples and reagents are flown through the nitrocellulose filter by manual pressure on the syringe; alternatevely, liquids can be actuated by a programmable syringe pump. A colorimetric detection enables naked eye results interpretation. Probe deposition by spotting allows for flexible combinations of different capturing agents and multiple diagnosis; furthermore, the very simple and cheap set-up make the syringe-based immunoassay on paper microarray a suitable diagnostic system for resource limited settings.

2. Materials and methods

2.1. Materials

Several filter papers were evaluated: Nitrocellulose Protran BA85 (0.45 μm porosity), Protran BA83 (0.20 μm porosity), PVDF Westran S (0.20 μm porosity) and Westran CS (0.45 μm porosity) from Whatman (GE HealthCare Europe GmbH, Milano, Italy) nitrocellulose BioTrace NT-Blotting, Biotrace NT-transfer (0.20 μm porosity) and PVDF-transfer (0.45 μm porosity) were from Pall Corporation (Pall Italia SRL, Milano, Italy). Biotrace NT-transfer (0.20 μm porosity) was selected for bioassay development. Swinnex® filter holders were from Millipore (Merck KGaA, Darmstadt, Germania). Luer-lok syringes were purchased from Becton Dickinson and Company (Becton Dickinson Italia, Milano, Italy). PBS (phosphate buffered saline), trizma base, bovine serum, tween 20, BSA (bovine serum albumin), and streptavidin–alkaline phosphatase from *Streptomyces avidinii* were purchased from Sigma (St. Louis MO). NBT/BCIP stock solution was purchased from Arrayit Corporation (Sunnyvale CA). HIV p24 antigen, hepatitis B virus (HBV) superficial antigen, corresponding capture and detection biotinilated-antibodies were kind gifts from Dia.Pro., Milano, Italy. The antibody pairs for sandwich immunoassays of HIV p24 and HBV antigens are mouse monoclonal antibodies providing limits of detection in the low pg/mL range when used in proprietary ELISA tests commercialised by Dia.Pro.

2.2. Spotting protocols

Capture antibodies for HIV p24 antigen and hepatitis B superficial antigen (1 mg/mL in PBS) were spotted on Biotrace-NT membranes using a SciFlexArrayer S5 spotter from Scienion (Berlin, Germany). For each replicated spot, 20 droplets (400 pL each) were deposited using a dot-pitch of 800 μm. Streptavidin–alkaline phosphatase (1 U/mL) was spotted as the positive control, and anti-α lactalbumin antibody was used as the negative control. After overnight binding, membranes were washed with PBS and passivated by BSA 2% w/v in PBS for 1 h.

2.3. Incubation protocols

The membranes were inserted on a 13 mm diameter Swinnex® syringe filter holder. Samples containing a mixture of HIV p24 antigen and HBV superficial antigen (HBV-SAg) at different concentrations from 0 to 50 ng/mL either in incubation buffer (Tris/HCl 0.05 M, pH 7.6, NaCl 0.15 M, Tween 20 0.02% w/v, BSA1% w/v) or in pure bovine serum were manually injected through the spotted membranes for 45 s. The sample was then allowed to stay in contact with the membrane for a total incubation time of 5 min. The membranes were then washed with 2 mL washing buffer (0.05 M Tris/HCl, pH 9, 0.25 M NaCl, 0.05% *v*/*v* Tween 20) and 2 mL PBS and then incubated for 10 min with a mixture of biotinylated detection antibodies for HIV p24 antigen and HBV-SAg (1 μg/mL each in incubation buffer) followed by colorimetric detection.

2.4. Colorimetric reaction and detection/quantification protocol

For colorimetric development, the arrays were incubated with streptavidin–AP, 0.1 U/mL in PBS for 10 min at room temperature and washed with PBS. They were then incubated in dark with an NBT/BCIP stock solution in 10 mL Tris–HCl, 0.1 M, pH 9.5 and 0.1 M NaCl for 10 min at 37 °C to speed up the colorimetric reaction and then washed with water. Room temperature incubation for 20 min provides similar results.

The results were visible with naked eye. Pictures of the arrays were taken using a stereo-microscope equipped with a Nikon SMZ 1500 camera and the Infinity Capture software from Lumenera Corporation. ImageJ software was used to analyse the images as follows: luminosities of spots and background were evaluated through the “Measure” function designing circular areas whose colour intensity was calculated by subtracting the backgroud around the spots. The relative intensity (*RI*) of the signals corresponding to the analytes and reported in the graphs was then calculated with the equation

$$RI = (A - N)/P$$

where *A* is the average intensity of analyte corresponding to spots; *N* is the average intensity of negative control spots and *P* is the average intensity of positive control spots. The relative intensities of 6–8 replicate spots were averaged.

2.5. Determination of the limits of detection

For detection limit experiments, the membranes were incubated with a mixture of HIV p24 and HBV-SAg at 100, 50, 20, 5, 2, 1, and 0 ng/mL in PBS or pure bovine serum. The membranes were then incubated with a mixture of biotinylated detection antibodies for HIV p24 and HBV-SAg followed by colorimetric detection and quantification as described above. Calibration curves (dose–response curves) were generated using a three-parameter equation in the OriginLab software. The detection limit was defined as the analyte concentration corresponding to a signal 3 times the standard deviation (SD) above the background signal as calculated from the linear range of the calibration curves.

3. Results and discussion

3.1. Selection of the porous membrane

Seven types of porous membranes, commonly used for western blotting, were tested to perform a colorimetric microarray assay for the detection of HBV superficial antigen (HBV-SAg) as described in Section 2. Relative intensity (*RI*) of spots was detected for a blank sample and for 10 ng/mL HBV-SAg. Fig. 1 reports the quantification of the average intensity of spots and the pictures of the microarray test for 10 ng/mL HBV-SAg performed on different membranes. The Pall nitrocellulose BioTrace membrane with a porosity of 0.2 μm was selected as the object of this study based on the higher intensity of signals, better morphology of spots and lower background intensity.