J Fluoresc (2015) 25:563–568
DOI 10.1007/s10895-015-1535-z

ORIGINAL PAPER

A Capillary Based Chemiluminscent Multi-Target Immunoassay

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Received: 24 November 2014 / Accepted: 20 February 2015 / Published online: 4 March 2015

Abstract Renewed interest in capillary format immunoassays has lead to increasingly costly and complex approaches to preparation and readout. This study describes a simple multi-target method based on a capillary platform using horseradish peroxidase (HRP) labelled IgG to visualize an antibody antigen complex. When goat-anti-human IgG was employed as the probe and human IgG as target, the system allowed detection of target to less than 1 ng/mL using a standard detection approach. The capillaries were read visually or with a commercial grade CCD camera. Multi-target detection was demonstrated using a model system of rat-anti-mouse, goat-anti-human and mouse-anti-rat IgG. These probes were encoded to different locations in the capillary, providing a simple inexpensive approach to achieve multi-target assays.

Keywords Diagnostic capillary · Immunological sandwich assay · Multiplexing assay · Detection limitatioin · Encoding immunoassay

Abbreviations

PCR	Polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
HRP	Horseradish peroxidase
TMB	3,3,5,5,-tetramethylbenzidine
CCD	Charge-coupled device
PBS	Phosphate Buffered Saline
BSA	Bovine Serum Albumin

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Introduction

Optical capillary format analytical systems and immunoassays have attracted renewed interest in the literature where they have been used to study proteins and divalent cations [1], multi-zone analysis of pesticides [2], multiple hormones in human serum [3], and screen for food pathogens [4, 5]. Further interest in the technique is shown by reports of specialized methods for patterning proteins to the inner surfaces of capillaries [6] and specialized readout of the capillaries [7]. As interest in the format has increased, there has been a corresponding trend toward increased cost and complexity. For example, capillaries have been used as waveguides, read out with pulsed dye lasers, and measured via chemiluminscent reporters [2, 3, 7, 8]. Other related methods include: a complementary metal oxide semiconductor (CMOS) biochip based on capillary reactors to detect pathogenic bacteria [9]; portable immunoassay systems based on a flow-through capillary format [10]; capillary confined bead arrays [11, 12]; and a variety of patents covering aspects of capillary based assays [13]. Early work on glass capillary immunosorbant assays provided a simple low-cost approach to detecting biologically important targets such as digoxin [14] or Herpes Simplex Virus [15]. This study, revisits the use of capillaries as a simple, low-cost platform.

Trends in diagnostic assays are toward micro-volume samples miniaturization and integrated devices, multiplexing and simple analysis [16–18] based primarily on two types of target molecules: nucleic acids and antigens and their immune response products [19–24]. Nucleic acids are detected by PCR or bDNA methods which provide high sensitivity and selectivity, but they are time-consuming and costly [19–21]. Immunological methods can provide more rapid analysis of antibodies or antigens than nucleic acid-based analyses of serum samples [13, 23, 24].

Enzyme-linked immunosorbent assays (ELISAs) are powerful and widely used tools providing high sensitivity and

selectivity [25, 26]. The 96-well format is a widely used platform to carry out ELISAs; however, this format requires considerable amounts of samples and reagents. Microarrays and biochips can perform automated testing for multiple targets but high cost limits the wide spread use of this technology. As a result, the development of improved, low-cost, multi-target assay platforms is still needed for clinical and research applications.

The aim of this paper is to present a multi-probe capillary based ELISA method which could achieve one-patient-one-capillary diagnosis with low sample consumption, short analysis time and simple operation. Probes are directly immobilized on the surface of the capillary through a controlled capillary action process leading to spatially encoded areas specific for individual probes or standards along the capillary. By using BSA blocked isolation, multiple assays can be achieved in one capillary, followed by visual or commercial grade digital camera readout.

Experiments

Reagents

Capillary tubes: Standard length (10 cm) glass capillary tubes with diameters of 1000 μm (SAMCO, VWR) were rinsed with sulfuric acid (95 %) prior to use. Reagents: Goat anti-human IgG (I1011, Sigma) and human IgG (I2511, Sigma), horseradish peroxidise (HRP) labelled goat anti-human IgG (A8667, Sigma), 3,3,5,5,-tetramethylbenzidine (TMB) (T5525, Sigma), PBS (P5368, Sigma), BSA (A7030, Sigma), Rat-anti-mouse IgG (Serotech Ltd) and mouse-anti-rat IgG (Serotech Ltd) were used as shown individual experiment. TMB was dissolved in deionized H_2O at the concentration of 0.15 mg/mL.

Procedure

Goat anti-human IgG, rat-anti-human IgG and mouse-anti-human IgG were diluted in PBS to a concentration of 10 μg/mL and used as probes. To bind the probes, the capillary was placed into the probe solution and the solution allowed to rise to a specific location by capillary action. The capillary was then placed horizontally at 37 °C for 4 h followed by a PBST (PBS buffer with 0.05 % Tween 20) rinse. A 1 % BSA solution was applied to block the unoccupied sites on the inner surface of the capillary and further rinsed with PBST. This process was repeated for each probe location for multi-probe studies. Details of the capillary preparation are illustrated in Fig. 1. The probe immobilized capillary tubes were then ready for use.

To standardize the assay, human IgG was diluted in PBS and used as a target. The probe immobilized capillary was put into the solution containing human IgG, placed horizontally at 37 °C for 30 min, and then rinsed several times with PBS. HRP labelled anti-human IgG (in PBS, 1:12,000 dilution) was applied to the capillary incubated for 30 min. at 37 °C and

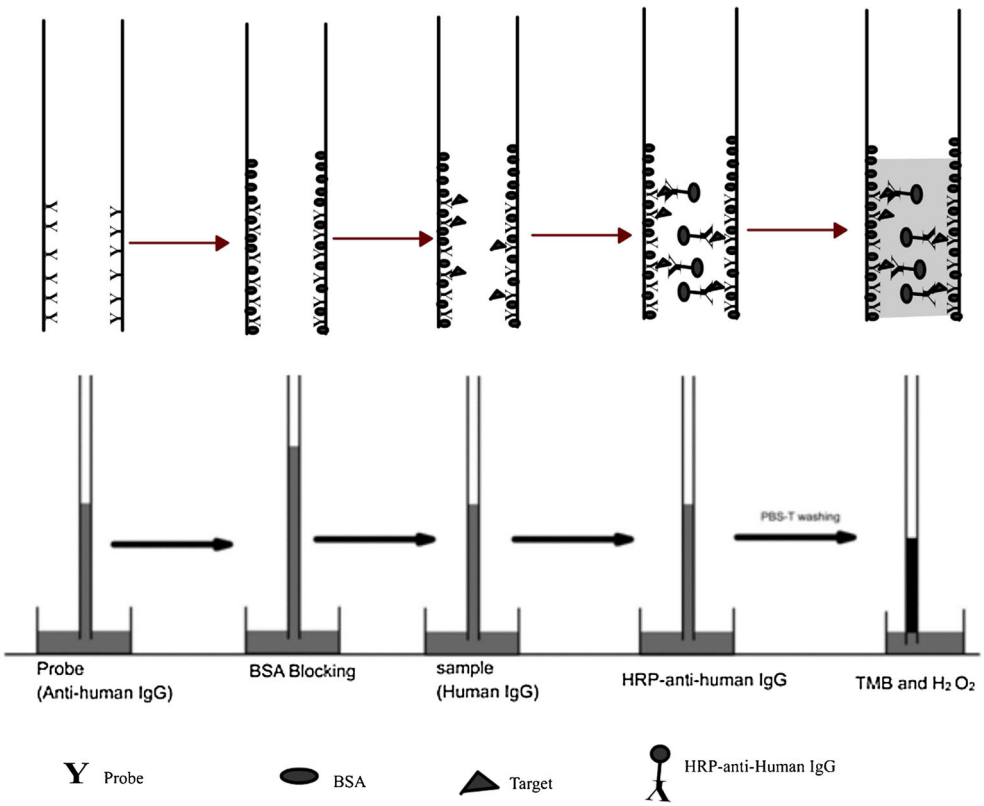

Fig. 1 Preparation diagram of capillary based immunoassay. The solution will climb up along the inner surface of the capillary because of the capillary action [18, 19]. The probe in the solution is adsorbed to the surface and then immobilized. After immobilization, washes, introduction of targets, reagents and developer was done under similar conditions

followed by PBS rinse. Finally, solutions of 1 % H_2O_2 and 0.1 mg/mL TMB were mixed just before the measurement and used to fill the capillary tubes before incubating horizontally at room temperature for 5 min.

Readout

Capillaries were photographed with a consumer grade CCD camera (P71, Sony, Japan) 5 min after loading the H_2O_2 - TMB solution. Images were analysed using *ImageJ* (Version 1.37v, Wayne Rasband, National Instututes of Health, http://rsb.info.nih.gov/ij) by separating the color channels within the image and using the red channel.

Results and Discussion

Format 1: Single Proble Capillary Assay

The simplest capillary bound assay consists of a single probe (anti-human IgG) immobilized throughout the capillary and visualized relative to a control (Fig. 2). A capillary without any probe but blocked with BSA did not change colour when treated with TMB and H_2O_2 following treatment with human IgG and HRP labelled anti-human IgG solution (Fig. 2a, capillary a). The capillary immobilized with probe (anti-human IgG) and blocked with BSA and treated with HRP labelled anti-human IgG did not change colour without being exposed to a target (human IgG) solution (Fig. 2a, capillary b). The

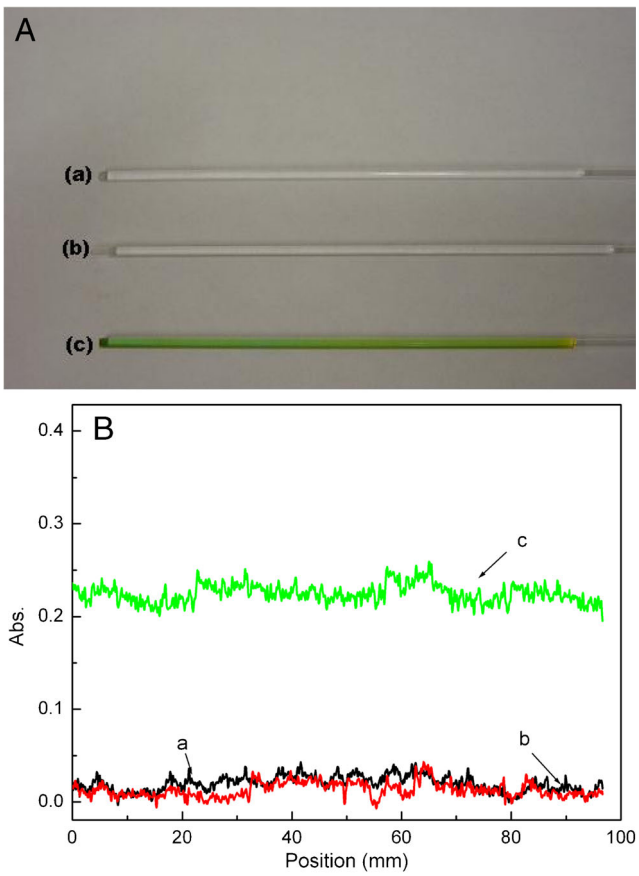

Fig. 2 Single target capillary based immunoassays. Control experiments of capillary based immunoassay (*A*) and the absorbance value for each capillary (*B*). Capillary (**a**) BSA+ HRP-anti-human IgG; capillary (**b**) Probe (anti-human IgG)+BSA+HRP-anti-human IgG; capillary (**c**) Probe (anti-human IgG)+BSA+ target (Human IgG, 10 μg/mL)+HRP-anti-human IgG. TMB was enzyme substrate

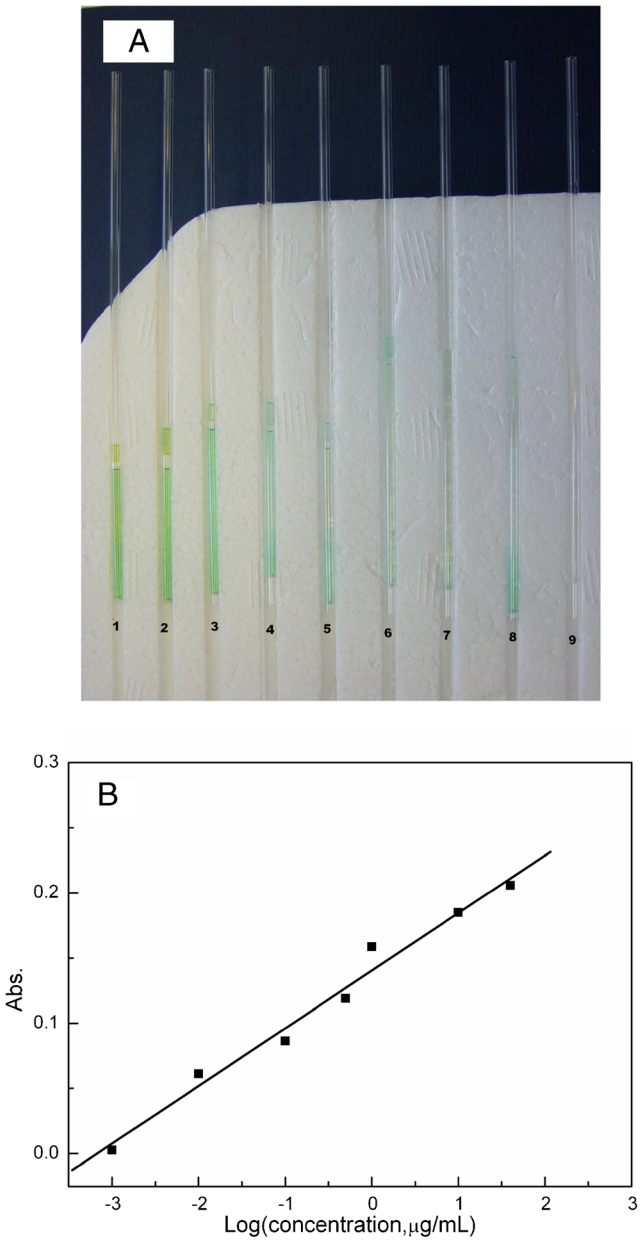

Fig. 3 Dose response study of capillary immunoassay. **a** Series target concentration tests of capillary based immunoassay. A set of capillaries were immobilized with goat anti-human IgG as probe and exposed to varying concentrations of target human IgG: 40 μg/mL (*1*), 10 μg/mL (*2*), 1 μg/mL (*3*), 0.5 μg/mL (*4*), 100 ng/mL (*5*), 10 ng/mL (*6*), 1 ng/mL (*7*) and (*8*), 0 ng/mL (*9*). TMB substrate concentrations were 0.1 mg/mL for (*1*) to (*7*), and 2.0 mg/mL for (*8*). All other conditions were the same. **b** Working curve of absorbance vs. concentration

capillary changed colour when reacted with TMB and H_2O_2 after being treated in sequence with Probe (anti-human IgG), BSA (to block non-specific binding), target (Human IgG) and HRP-anti-human IgG (Fig. 2a, c). The absorbance of the positive capillary is significantly higher than the blank and control (Fig.2b). This experiment proved the capillary was a suitable matrix to carry out assays in a simple format driven by capillary action.

To investigate the sensitivity of the capillary bound assay, further experiments were carried out while changing the target (human IgG) concentration (Fig.3a). Capillaries treated with solutions containing varying amounts of target IgG (1 ng/mL to 40 μg/mL) exhibited a positive visually detected response (Fig. 3a, capillaries 1 to 8) proportional to a target concentration. Using digital camara detection, the working curve of concentration and absorbance (Fig. 3b) indicated a detection limit of ~1 ng/mL. The observed sensitivity of this assay is sufficient to detect typical concentrations of many known target molecules in blood [27–29].

Format 2: Multi-probe capillary Assay

A multi-zone assay was constructed such that a probe was immobilized to three different zones within the capillary, separated by blank regions produced by BSA blocking (Fig. 4). Three zones were produced in the capillary by immobilizing goat-anti-human IgG in three bands separated by BSA to target human IgG and detected with HRP-labelled anti-human IgG. The results indicated that all three bands successfully reported the presence of target and these bands were separated by the blank zones (Fig.5a, Capillary a) relative to a control capillary (Fig. 5a, Capillary b) and the absorbance of the positive zones were higher than that of the control capillary (Fig. 5b). The zones were still detectable after an observation period of 30 min, although the blue solute slightly diffused. In theory, the number of encoded bands will depend on the length of capillary, the distance between encoding and blocking bands, and the rate of diffusion. These results indicate that probe bands can be readily produced to create a multi-zone assay and that these bands may be used to encode multiple targets or to provide a standard series within the capillary.

To study the specificity of the multi-probe assay, three different probes encoded capillary (Fig. 6a, Capillary b) was developed consisting of rat-anti-mouse IgG, goat-anti-human IgG and mouse-anti-rat IgG to zones 1, 2, and 3, respectively, as shown in Fig. 4b. Another capillary was encoded with goat anti-human IgG at three different zones (zones 1, 2, and 3) and used as a control (Fig. 6a, Capillary a). A third capillary was not encoded with any probe but was blocked with BSA only and used as a negative control (Fig. 6a, Capillary c). Human IgG in PBS was applied as target and followed by treatment with HRP labelled anti-human IgG and substrate. Results (Fig. 6a) clearly show that the "Capillary a" demonstrated three reaction zones, while "Capillary b" only the corresponding zone responded to the respective antigens, i.e., anti-human IgG. The absorbance of the positive zone in "capillary b" exceeded those of the negative zones (Fig. 6b) indicating that

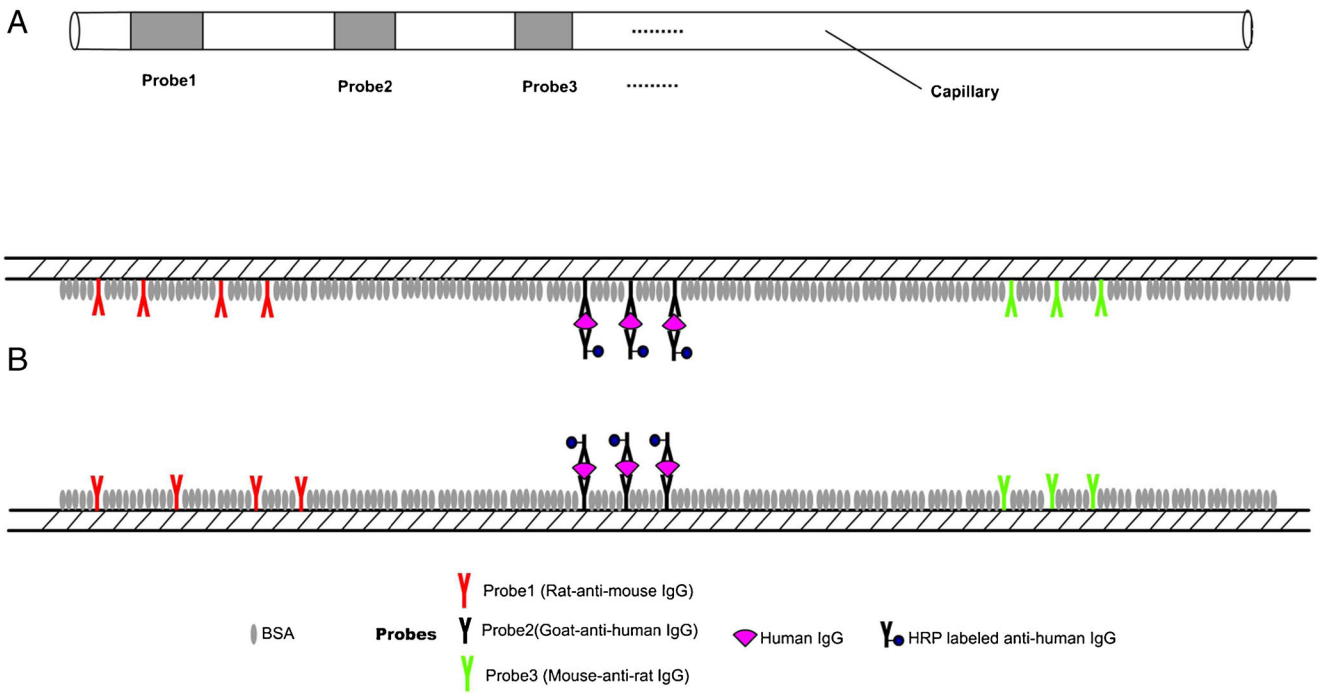

Fig. 4 Schematic of a multiprobe capillary immunoassay. Probes are immobilized to different bands within the capillary and separated by the blank bands blocked with BSA (**a**); the probes capture the targets and the reporter to give out the position of a positive band (**b**)

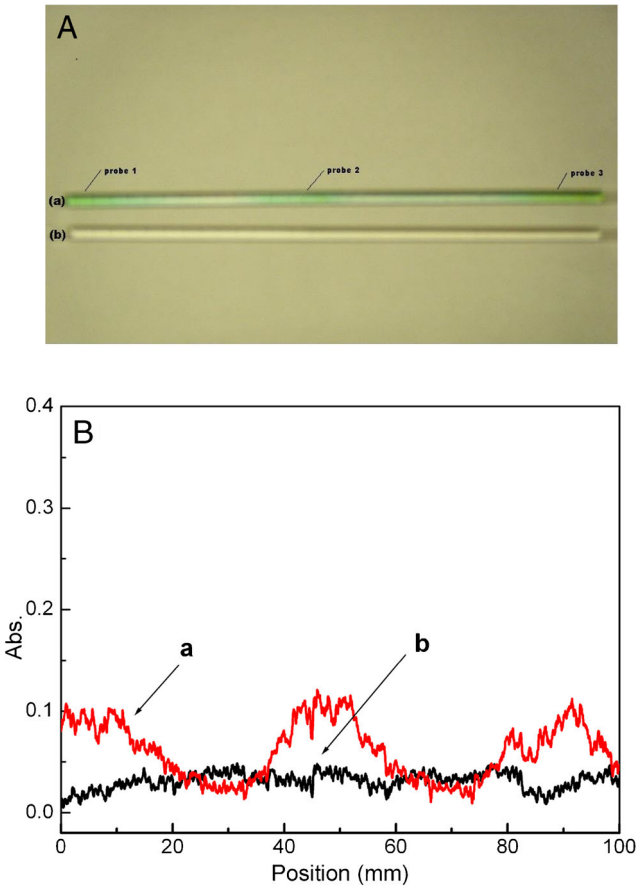

Fig. 5 Multi-zone capillary assay. **a** The appearance of positive (*a*) and control (*b*) capillaries after the reaction with HRP substrate. Probe 1= probe 2=probe3=goat-anti-human IgG; human IgG was target for detection; the control capillary was not immobilized with probe; other conditions were the same. **b** The absorbance measurements along the length of each capillary

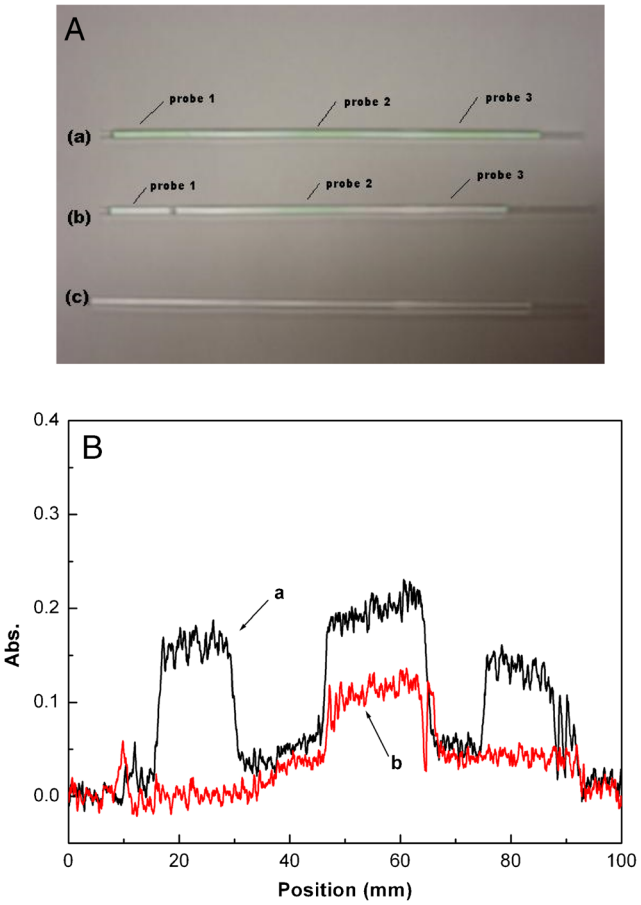

Fig. 6 Multi-zone multiprobe immunoassay. **a** Image of three capillaries: (*a*) Probe 1=probe 2=probe 3=Goat-anti-human IgG; (*b*) probe 1=Rat-anti-mouse IgG, probe 2=Goat-anti-human IgG, probe 3=Mouse-anti-rat IgG; (*c*) control. All the capillaries were blocked by BSA and the human IgG in PBS was target at the concentration of 0.1 μg/mL. TMB was the substrate. Other conditions were the same. **b** The absorb value of capillary *a* and *b*

only the anti-human IgG probe captured the target human IgG while the rat-anti-human and mouse-anti-rat IgG did not react with the target. The results obtained from this model system indicate that a similar strategy could be used to diagnose multiple disease targets. This capability could be used to detect multiple serotypes as well as multiple antigens or their immune responses.

Conclusions

In this study, a low cost, sensitive, multi-probe capillary based immunoassay has been demonstrated in a set of model systems. In this method, the probe immobilized capillary achieved good detection limits and was capable of detecting multiple targets. The method has a number of advantages: the required materials are relatively inexpensive compared to PCR and microarrays and had a similar level of sensitivity to dye laser excited fluorescence detection [2]. Reagent consumption is low and visual or consumer grade digital camera detection represents a low cost alternative to ELISA plate readers. As previously demonstrated [15]. the capillary procedure may allow simple point of care diagnosis and based on the current work multiple targets can be detected. Preparation of the tubes is simple, does not require photochemical patterning [6] and should be readily scalable for manufacturing. Coated capillaries could be produced by pumping antibody solutions through long capillary sections, these sections could then be cut and assembled into made-to-order diagnostic capillaries. The high surface to volume ratio of the capillary is advantageous for rapid development of the assay [15]. While conventional ELISA reagents were used in the current study, other reporting methods could be adopted which allow the assays to store: quantum dots, fluorophores, and magnetic particles. As such, this study has demonstrated a portable, simplified muti-probe alternative to more costly systems reported in the literature.

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