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ARTICLE TYPE

# Stacking Flow Immunoassay for the Detection of Dengue-Specific Immunoglobulins in Salivary Fluid

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Paper-based immunoassay, usually in the form of lateral flow test, is currently the standard platform for home diagnostics. However, conventional lateral test is often complicated by severe non-specific adsorption of detector particles when applied to test samples containing salivary fluid. It is believed that a high concentration of proteinaceous substances in salivary fluid causes the particle aggregation and adhesion. In this study, we have developed a stacking flow platform for single-step detection of target antibody in salivary fluid. The stacking flow circumvents the need for separate sample pre-treatment, such as filtration or centrifugation, which is often required prior to testing saliva samples with paper-based immunoassay. This is achieved by guiding samples and reagents to the test strip with different paths. By doing so, salivary substances that interfere with the particle-based sensing system are removed before they make contact with the detection reagents, which greatly reduces the background. In addition, the stacking flow configuration enables uniform flow with a unique flow regulator, which leads to even test lines with good quantification capability, enabling the detection of ~ 20 ng/mL  $\alpha$ -fetoprotein in the serum. We have successfully applied the stacking flow device to detect dengue-specific immunoglobulins that are present in salivary fluid.

## Introduction

In spite of great advances in point-of-care technology in the last few decades, paper-based immunoassays, often in the form of lateral flow test, remain the mainstream product in the market for home diagnostic tests.<sup>1</sup> These tests often rely on antigen-antibody interactions to detect targets of interest in bodily fluids, such as serum, blood, or urine. Depending on the assay format, either antigen or antibody is immobilized on the paper substrate as the capture agent. Targets of interest bind to immobilized capture agent, resulting in visually distinguishable lines or spots generated by colorimetric, fluorescent, or enzymatic conjugates. Currently, a wide selection of lateral flow test kits is available, ranging from well-known pregnancy test and ovulation test for personal healthcare, to highly specialized tests for pathogen genotyping<sup>2, 3</sup> and meat speciation.<sup>4, 5</sup> The greatest advantage of paper-based immunoassays lies in its simplicity. Capillary forces provided by the paper are sufficient to draw the sample to the test strip, and reagents are often stored in the dry form at room temperature, which eliminates the need for external pumping mechanisms for fluidic manipulation, and refrigeration units for device storage. These have led to self-contained tests that are easy to conduct by inexperienced users in low-resource settings. In addition, lateral flow tests usually deliver colorimetric readouts that can be identified by unaided eyes, which significantly reduces costs associated with instruments.

To expand the applicability of paper-based systems to more complex assays, a number of groups have explored the potential of paper for complex fluidic manipulation. Whitesides and co-

workers have pioneered the paper microfluidics by introducing hydrophobic barriers to the paper substrate using various techniques<sup>6-8</sup> for colorimetric assays, such as protein and glucose sensing,<sup>9, 10</sup> as well as electrochemical sensing.<sup>11</sup> Very intricate fluidic control has been demonstrated on the paper microfluidic platform with the ability to route the liquid in 3 dimensions in order to distribute multiple reagents to different locations.<sup>12</sup> Yager and co-workers have developed a two-dimensional (2D) paper network with controlled timing to perform autonomous multistep assays,<sup>13-16</sup> and demonstrated its application in malaria antigen detection.<sup>17</sup>

Despite its popularity, the performance of paper-based immunoassays is frequently compromised by the formation of aggregates between conjugates and samples, which prevents the labeled analytes from reaching the test zone.<sup>18</sup> This issue is particularly evident with saliva samples. Salivary fluid is an important source of biomarkers, and is useful for rapid point-of-care diagnostics. The immunoglobulins (*e.g.* IgGs and IgMs) found in salivary fluid are directly related to those in blood.<sup>19</sup> Human saliva also carries lymphocytes and plasma cells, which may also serve as biomarkers.<sup>20, 21</sup> Steroids are passively carried into saliva, and their quantities are closely correlated to plasma levels.<sup>20</sup> In addition, salivary fluid can be easily collected in a completely non-invasive manner, leading to higher patient compliance and willingness to take the test. As a result, antigen-antibody tests based on salivary fluid provide a simple and easy way of assessing both oral and systemic diseases.<sup>20</sup> However,

unlike the other fluid specimens, salivary fluid cannot be applied directly to commercially available blood or urine lateral flow test strips because it causes the detector colloidal particles, which are referred to as conjugates, to non-specifically adhere to the nitrocellulose membrane.<sup>21</sup> This peculiar behavior is believed to be caused by the presence of high concentrations of mucin and other proteinaceous and viscous substances that adhere to the paper and aggregate the conjugates. Furthermore, compared to serum, the antibody levels in saliva are lower by a few orders of magnitude.<sup>19</sup> Therefore, in order to obtain accurate results, a large volume of saliva sample is required for each test. However, conventional lateral flow devices are often not designed to handle large sample volumes. If too much sample is applied, it will result in the overflow of the fluid and flood the test strip.

One solution to the aforementioned problem is to collect and pre-treat the saliva samples in a separate step before introducing them to the lateral flow device.<sup>22</sup> However, the separate sample preparation step would defeat the purpose of having a self-contained test and render the device less user friendly. Another solution is to introduce the sample and conjugates from two separate paths, one of which contains materials and reagents that remove the substances causing the non-specific adhesion. However, such an approach requires multiple user activation steps at specific time points,<sup>18, 23</sup> hence, it is undesirable for home test applications. The timed 2D paper microfluidic network<sup>13</sup> enables autonomous delivery of liquid reagents from multiple flow paths, which is a preferred feature in this case. However, we notice that 2D network sometimes results in non-uniform flow across the test strip and leads to uneven test lines<sup>24, 25</sup> due to the laminar nature of the flow in the paper.<sup>26</sup> In this study, we created a stacking flow device for detecting targets of interest in salivary fluid. Samples and reagents were introduced from multiple stacked but separated flow paths in a single user activation step. The sample flowed through a matrix of fiber glass, which removed proteinaceous substances and particulates in salivary fluid before the sample reacted with the reagent. By doing so, the non-specific adhesion caused by salivary substances was significantly reduced. Furthermore, the stacking flow configuration regulated the flow in the test strip, ensuring even test lines for reliable quantification. The stacking flow design introduced minimal complexity to the conventional lateral flow construct, and could be easily adapted to existing manufacturing practice. We successfully applied the stacking flow platform to detecting dengue-specific IgG in salivary fluid, as part of the test to distinguish primary and secondary dengue infection.<sup>27</sup>

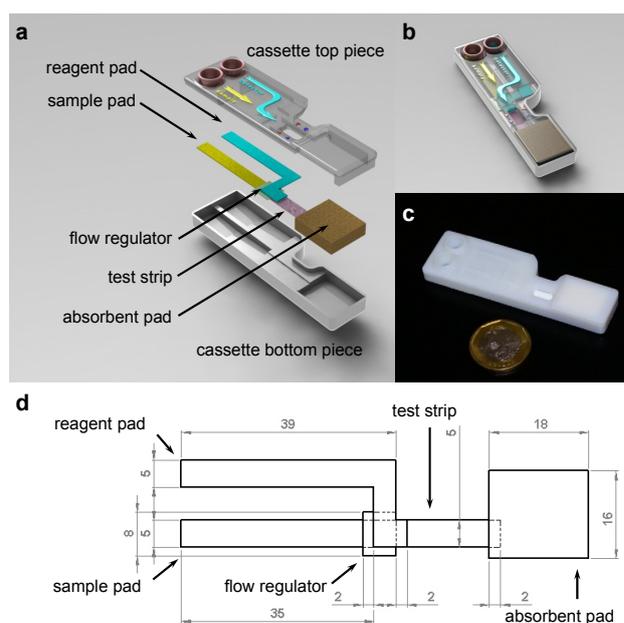
## Methods and Materials

### Device design and prototype

The stacking flow device consisted of a test strip, a sample pad, a reagent pad, an absorbent pad, and a flow regulator (Fig. 1). The test strip (Hi-flow plus 75 membrane cards, Merck Millipore, Massachusetts, USA) was comprised of nitrocellulose for protein immobilization. Sample and reagent pads were made of glass fiber (Merck Millipore, Massachusetts, USA), and the absorbent pad was made of cellulose (Merck Millipore, Massachusetts, USA). The nitrocellulose test strip was cut into the required

dimensions manually with a roller cutter. All other sorbent materials were cut into desired shapes by a CO<sub>2</sub> laser cutter (Epilog Laser, Colorado, USA). The flow regulator made of liquid impermeable film (film with adhesives for PCR plate, 4titude, Surrey, UK) was cut to the required dimensions using an automated stylus cutter (Pazzles, Idaho, USA). All parts were assembled according to Fig. 1d. The widths of the sample pad, reagent pad and the test strip were all 5 mm. The sample pad was positioned with 2-mm overlap with the test strip. The flow regulator, a liquid impermeable membrane used to separate streams,<sup>26</sup> was then placed above the sample pad and the test strip. The flow regulator covered the sample pad by 2 mm and the test strip by 4 mm. The reagent pad was then laid over the flow regulator with an overlap of 4 mm. The end of the reagent pad extended from the flow regulator and overlapped the test strip by 2 mm. Two pieces of absorbent pads sandwiched the test strip downstream, and overlapped with the test strip by 2 mm.

The cassette was designed to house the test assembly, and to apply the right amount of pressure at the overlapping regions to ensure good contact between various materials. The cassette was designed with Solidworks and prototyped with a 3D printer (Stratasys, Rehovot, Israel). Two reservoirs were included in the design to hold samples and reagents, considering the relatively large sample volume required due to the low analyte concentration in salivary fluid.



**Fig. 1** Device for stacking lateral flow immunoassay. (a) The exploded view of the device. The test assembly consists of sample pad, reagent pad, flow regulator, test strip and absorbent pad. The test assembly is housed in the cassette. (b) The assembled stacking flow device. (c) Photograph of the stacking flow device prototyped with a 3D printer. (d) Schematic of the test strip assembly. All dimensions are in millimeter (mm).

### Test strip preparation

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Two customized buffers were used in this study. 1× flow buffer contained 1× phosphate buffered saline (PBS) (First base technology, Singapore) supplemented with 0.1% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween 20. 1× conjugate buffer contained 2.5 mM of Tris (pH = 7–7.5) (First base technology, Singapore) supplemented with 5% (w/v) sucrose and 0.5% (w/v) BSA.

To prepare the test strip for the detection of dengue-specific IgG, type 2 dengue antigen (Microbix Biosystems, Ontario, Canada) were diluted 4-fold from stock to a final condition with 0.025% (w/v) sodium dodecyl sulfate (SDS) and 25 mM of Tris (pH = 7–7.5). Control antibody (Goat anti-human IgG, Arista Biologicals, Pennsylvania, USA) was diluted to 0.4 mg/mL with a buffer containing 0.01% (w/v) SDS and 5 mM of phosphate buffer (pH = 7–7.5). The dengue antigen was dispensed into the test line at 1.5 μL/cm with an automated liquid dispenser (Biodot, California, USA). The control line was dispensed 5 mm downstream of the test line using the same settings. The test strips were then dried in vacuum for 30 min for protein adsorption. After that, the test strips were blocked with blocking solution (Candor Biosciences, Wangen, Germany) for 30 min, and washed with 5 mM of phosphate buffer (pH = 7–7.5). Lastly, test strip were dried again in vacuum overnight and stored in low humidity until use. To illustrate the versatility of the stacking flow platform, we demonstrated both liquid and dry conjugate approaches for target detection. In the liquid conjugate approach, the antibody-conjugated detector particles were stored in the solution, and they were mixed with the sample right before the test. In the dry conjugate approach, the antibody-conjugated detector particles were pre-dried on the conjugate pad, which in our case was the reagent pad. Upon the application of the sample or buffer, the liquid would hydrate the particles and release them from the conjugate pad so that particles could flow in the test strip.

The detection of dengue-specific IgG was conducted using the liquid conjugate approach. The liquid conjugate was comprised of protein G-conjugated 40-nm gold nanoparticles (Arista Biologicals, Pennsylvania, USA) at 0.5 optical density (OD) in 1× flow buffer.

The test strips for α-fetoprotein (AFP) detection were prepared the same way except the capture antibody (Arista Biologicals, Pennsylvania, USA) concentration was 2 mg/mL. The AFP detection was conducted using the dry conjugate approach. To prepare the conjugate pad, the detector antibody (Thermo Pierce, Illinois, USA) was labeled with biotin (Lightning Link® Biotin Type B, Innova Bioscience, Cambridge, UK), and a final concentration of 12.5 μg/mL of biotinylated antibody was mixed with streptavidin-conjugated 40-nm gold nanoparticles (Arista Biologicals, Pennsylvania, USA) at 0.5 OD in 1× conjugate buffer. 40 μL of the conjugate mixture was applied on the reagent pad and allowed to dry overnight in ambient environment at room temperature.

### Image acquisition and analysis

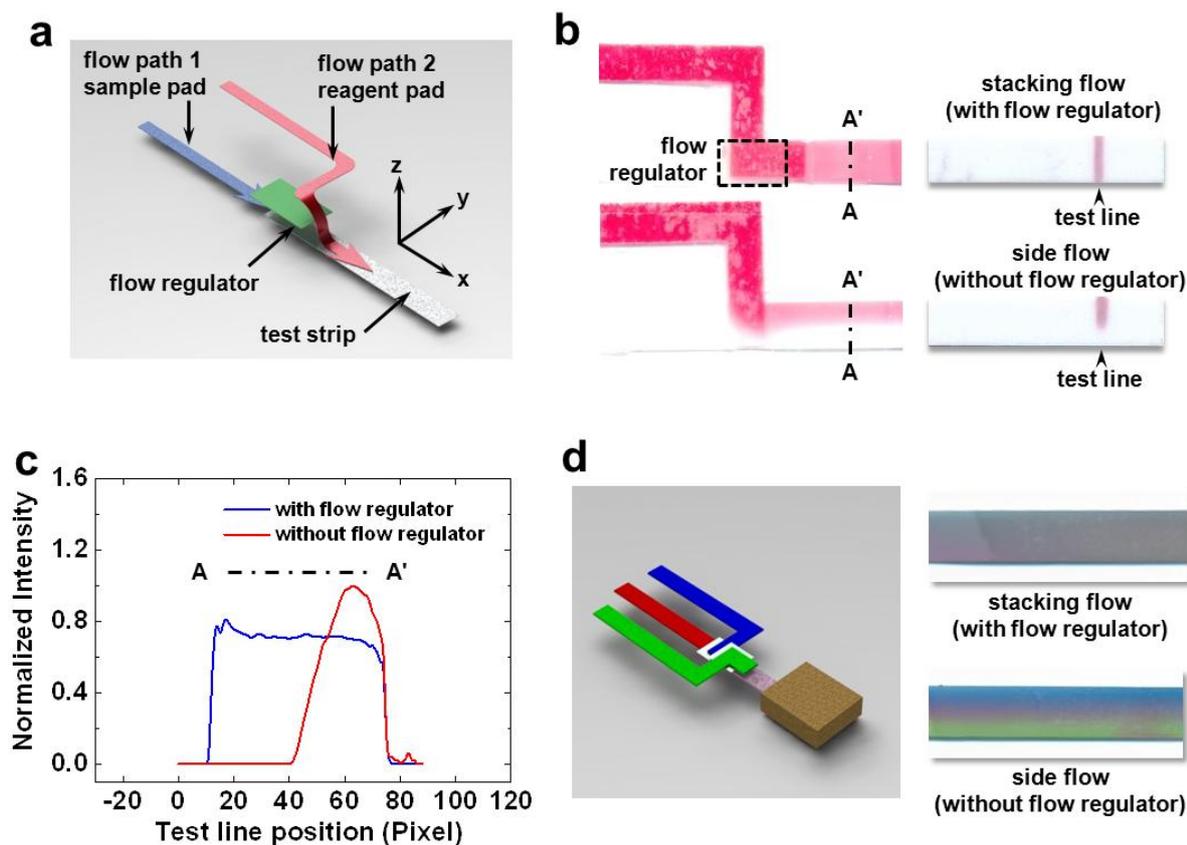
All images were acquired using a high-resolution scanner. Image analysis was performed using ImageJ (National Institute of Health, Maryland, USA). The region of interest was defined as the area of the test line generated by the sample containing the highest amount of analytes. The same region of interest was applied to other test strips even though the actual visible test line might be thinner.

## Results and Discussion

### Flow regulation

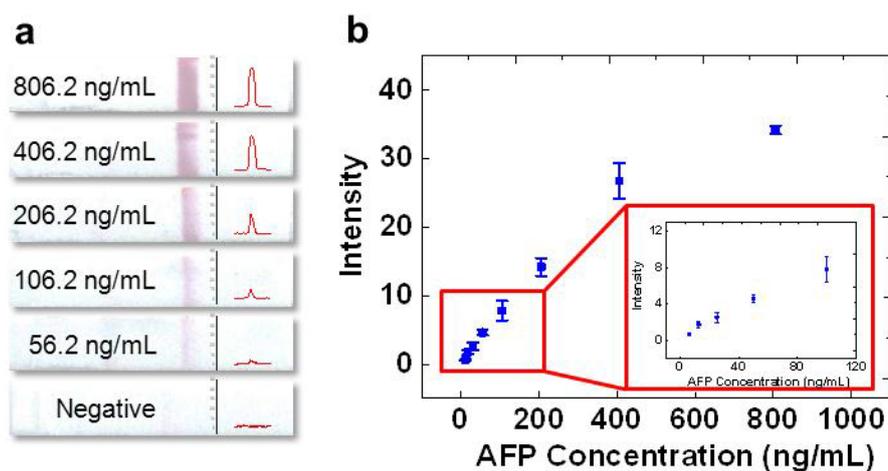
When multiple streams were introduced into the test strip in a conventional 2D paper microfluidic network, at most one stream would flow in the same direction as the test strip (direction *x* in Fig. 2a). The other streams would inevitably enter the test strip at a certain angle from the side of the test strip because of spatial restriction. Due to the laminar nature of the flow in paper microfluidic network,<sup>26</sup> liquid from different streams would flow in layers in the test strip, resulting in non-uniform composition in the direction perpendicular to the flow (direction *y* in Fig. 2a), which would ultimately lead to uneven test lines downstream<sup>24, 25</sup> (Fig. 2b). Our stacking flow design would circumvent the problem by stacking flow paths normal to the test strip (direction *z* in Fig. 2a), thereby allowing all streams to enter in the same direction. In a two-path stacking flow configuration (Fig. 2) for lateral immunoassay, the sample pad was placed at the bottom and in direct contact with the test strip. The reagent pad was located above the sample pad with a flow regulator inserted in between. The flow regulator was made of a liquid impermeable film. It has multiple functions, including separating streams in the two flow paths, guiding the flow from the second path to enter the test strip in the same direction as the first path, adjusting the distance between the entry points between the two streams, and controlling the overlapping length between the reagent pad and the test strip.

To illustrate the characteristics of the stacking flow, we observed the liquid streams in the test strip by applying water on the sample pad, and red food dye on the reagent pad (Fig. 2b). With stacking flow, the food dye entered the test strip in the same direction as the water, and encompassed the entire width of the test strip. In contrast, the conventional side flow exhibited typical laminar behavior. The food dye flowed in a layer close to the top edge of the test strip where it entered, and pushed the water stream to the bottom edge of the test strip. The flow non-uniformity is clearly evidenced in Fig. 2c, which shows the flow profile of the food dye at the cross section *AA'*. To evaluate the effect of flow uniformity on the test line, 100 μL of protein G-conjugated 40-nm gold nanoparticles at 0.1 OD in 1× flow buffer was introduced to the test strip from the reagent pad, and 100 μL of 1× flow buffer was applied to the sample pad. The test line was created by dispensing 0.4 mg/mL of goat anti-human IgG at 1.5 μL/cm. The stacking flow resulted in a clear test line across the test strip, whereas the conventional side flow led to an uneven test line because the majority of gold nanoparticles flowed through the test strip in a layer close to the top edge (Fig. 2b).



**Fig. 2** Characterization of stacking flow. (a) The schematic illustration of flow directions in stacking flow immunoassay device. The flow regulator inserted between the sample pad and the reagent pad ensures streams in the two paths enter the test strip in the same direction in order to achieve uniform flow. (b) Comparison of flow characteristics in stacking flow and conventional 2D side flow. The stacking flow generates a uniform stream in the test strip, leading to an even test line. In contrast, the side flow exhibits typical laminar layers, resulting in an uneven test line. (c) The flow intensity profile along the cross section AA'. (d) Stacking flow platform with 3 paths. The laminar features are only observed with the side flow, but not with the stacking flow platform.

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**Fig. 3** Detection of AFP in serum with stacking flow immunoassay platform. (a) Images and test line intensity profiles of the test strip from the AFP dilution series tested with stacking flow. (b) Graphs of average test line intensity versus AFP concentration. A strong correlation is observed, indicating good quantification capability.

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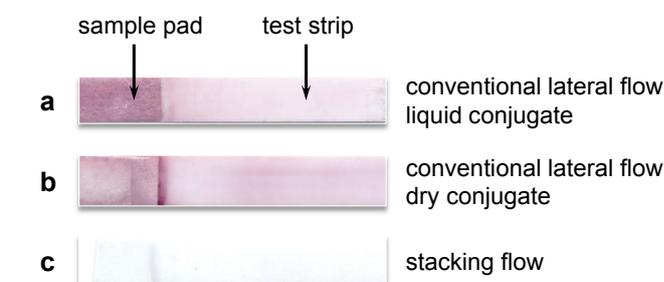
The stacking flow design distinguished itself in a number of aspects from the dual-path immunoassay device<sup>18, 23, 28, 29</sup>, which is a commercially available immunoassay platform that has successfully implemented the design of multiple input streams. Firstly, the dual path immunoassay device would only allow a maximum of two flow paths. In comparison, the stacking flow device was able to accommodate two or more flow paths as necessary. As shown in Fig. 2d, three food dyes were introduced from three different paths. The food dyes flowed uniformly in the stacking flow configuration. In comparison, characteristic laminar layers were observed in the conventional side flow construct. Secondly, in the dual path immunoassay device, the two paths would only intersect at the test zone. In the stacking flow device, flow paths were free to enter the test strip at arbitrary locations. The reaction time between analytes and conjugates was adjusted by controlling the distance between the entry points. If the reaction between analytes and conjugates was not desirable before the capture of analytes at the test zone, the entry point of the reagent pad would be positioned close to the test zone. Thirdly, in the dual path immunoassay device, liquid in the second path could only be introduced after the liquid in the first path had completely migrated through the test zone, which would require multiple user activation steps at specific time points. In the stacking flow device, liquids in all paths flowed concurrently in the same direction.

#### Quantification with stacking flow platform

In order to evaluate the performance of stacking flow for paper-based immunoassay, we first performed a model assay that detected AFP in serum. AFP is an indicator of liver function, and is often used as a tumor marker to help with the diagnosis of liver cancer.<sup>30, 31</sup> Although specifically designed to handle salivary fluid, stacking flow could be widely applicable to many other types of sample matrix, including serum and blood. The serum used in this study was first validated by traditional enzyme-linked immunosorbent assay (ELISA), and showed that trace amount (6.2 ng/mL) of AFP was present. Next, a two-fold serial dilution of AFP (CalBioReagent, California, USA) was spiked into the serum. The stacking flow immunoassay test strip was assembled according to Fig. 1. A dry conjugate approach was employed for AFP detection. The detector conjugate was dried on the reagent pad, and assembled into the device. 100  $\mu$ L of serum containing various amounts of AFP was applied to the sample pad first, and 100  $\mu$ L of 1 $\times$  flow buffer (see Methods and Materials) was applied to the reagent pad immediately after that. Both liquids flowed concurrently in the test strip. After the completion of the reaction, the test line became visible (Fig. 3a), and the peak intensity of the test line decreased with decreasing amount of AFP. The average intensity of the test line was then plotted as a function of the AFP concentration (Fig. 3b). A strong positive correlation was observed, which indicated good quantification capability of the stacking flow immunoassay. The test line was observable by unaided eyes down to 56.2 ng/mL of AFP, and signal from 18.7 ng/mL of AFP was distinguishable from the negative control with computer-assisted analysis. Normal physiological AFP range was < 10 ng/mL, which would appropriately appear as a negative result on the stacking flow platform. Investigation of hepatocellular carcinoma (HCC) is

recommended for an AFP value of > 20 ng/mL. A cut-off AFP value of 400 ng/mL is usually utilized as a confirmatory test for HCC diagnosis in the presence of solid lesion in the liver<sup>32</sup>. The diagnostic range of AFP mentioned above fell within the dynamic range of the stacking flow platform, suggesting the potential application of stacking flow for cancer biomarker detection.

As mentioned earlier, the key design requirement for stacking flow was the ability to remove substances in salivary fluid that interfere with the nanoparticle-based sensing system in paper-based immunoassays. Furthermore, the entire analysis should only require a single-user activation step, and be able to provide uniform flow and even test lines. We first investigated whether stacking flow would reduce the background resulted from the non-specific adhesion of gold nanoparticles in salivary fluid. To collect saliva sample, the test subject was instructed to rinse mouth with 1 mL of water, and spit into a sample collector. We first examined the background on conventional lateral platform using both liquid and dry conjugates. For the liquid conjugate, gold nanoparticles of a final OD of 0.5 were mixed with 200  $\mu$ L of saliva in 1 $\times$  flow buffer. For the dry conjugate, 40  $\mu$ L of gold nanoparticles in 1 $\times$  conjugate buffer was dried on the glass fiber and assembled onto the lateral test strip. In the case of liquid conjugate, a majority of gold nanoparticles adhered to the sample pad, and could not even reach the test strip (Fig. 4a). In the case of dry conjugate, although a large portion of gold nanoparticles migrated from the conjugate pad to the test strip, they ended up sticking to the test strip, which significantly increased the background (Fig. 4b). In contrast, on the stacking flow platform, the sample passed through a fiber glass pad, which effectively removed the proteinaceous substances and particulates in the salivary fluid, before reacting with detector reagent from the other path. As a result, all particles flowed through the test strip with a negligible amount of residue at the interface between the reagent pad and the test strip, and the non-specific background was kept to the minimum (Fig. 4c).

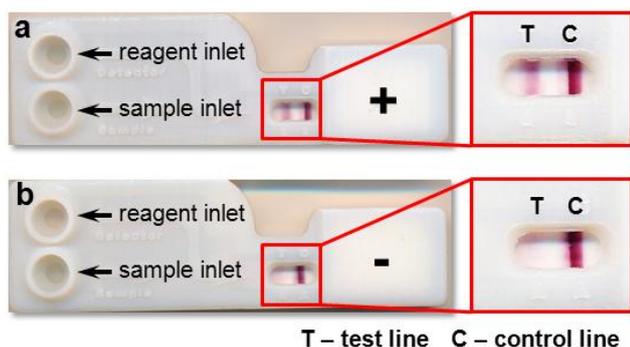


**Fig. 4** Comparison of background between conventional lateral flow and stacking flow platform tested with samples containing salivary fluid. (a) Conventional lateral flow with liquid conjugate. The majority of gold nanoparticles stick to the sample pad. (b) Conventional lateral flow with dry conjugate. A large portion of gold nanoparticles adhere to the test strip. (c) Stacking flow significantly reduces the background caused by non-specific adhesion due to the incompatible salivary substances. All test strips are washed by flowing 100  $\mu$ L of 1 $\times$  flow buffer.

### Detecting dengue IgG with stacking flow platform

Dengue virus is the leading cause of death in the tropics and subtropics. Infection by dengue virus causes dengue fever.<sup>27, 33, 34</sup> It is believed dengue patients with secondary infection (those who have been infected with other serotype of dengue viruses previously) are at a higher risk of developing into more severe conditions, such as dengue haemorrhage fever or dengue shock syndrome.<sup>35, 36</sup> Detecting dengue-specific IgGs offers a valuable tool to distinguish primary and secondary dengue infection because IgGs are only present in secondary infection, but not in primary infection, during the acute phase (i.e. the first 5 days) of the disease.<sup>27</sup>

To prepare dengue IgG-positive and -negative samples, salivary fluid was collected from healthy individuals with no history of dengue infection. The test subject was instructed to rinse mouth with 1 mL of water, and spit into a sample container. Dengue IgG-positive and -negative serum samples were validated using conventional ELISA. The sera were spiked into salivary fluid with 800-fold dilution to match the typical antibody concentration in saliva.<sup>37, 38</sup> The dengue IgG stacking flow test strip was prepared with both a test line and a control line (Fig. 5). 400  $\mu$ L of spiked saliva sample was mixed with 1 $\times$  flow buffer, and introduced from the sample inlet. 200  $\mu$ L of liquid conjugate containing protein G-conjugated 40-nm gold nanoparticles of 0.5 OD in 1 $\times$  flow buffer was introduced from the reagent inlet. After 20 min, the signals were read from the observation window. For both positive and negative saliva samples, the control line appeared, indicating that the test was valid. However, the test line only appeared in the positive sample, signaling the presence of dengue-specific IgG. Unlike many other multistep lateral flow tests whereby samples and reagents were added sequentially at specific time points with several user activation stages, the stacking flow immunoassay platform required only one user activation, and both the sample and reagents were added at the same time in a single step. Both the sample and reagents reacted and flowed concurrently in the test strip. The single-step procedure significantly improved the ease of usage of the test kit.



**Fig. 5** Images of stacking flow immunoassay device for the detection of dengue-specific IgG with salivary fluid. In the positive sample, both the test line and the control line are visible. In the negative sample, only the control line is observed.

### Conclusions

We have demonstrated successful detection of dengue-specific

IgG in salivary fluid using the stacking flow immunoassay platform. The stacking flow platform offers single-step detection of targets in salivary fluid, while significantly reducing the background by removing salivary substances that interfere with the particle-based sensing system. As a result, gold nanoparticles would not non-specifically adhere to the test strip. Furthermore, the stacking flow configuration enables uniform flow across the test strip, generating even test lines with ease of quantification. Although designed specifically for saliva sample, the stacking lateral platform can be widely applicable to other types of common sample matrix, such as blood, serum and urine. We believe the stacking flow platform provides a versatile and improved performance to paper-based immunoassay. The detection of IgG alone would shed some light on the history of infection and immunity to the disease. However, it is not able to provide a definitive diagnosis on the current status. Therefore, we will look into the detection of dengue-specific IgM and IgA in saliva<sup>39, 40</sup> in future work. In combination with the existing dengue-specific IgG test that we have demonstrated in this study, we would be able to obtain more comprehensive information to diagnose dengue and distinguish primary and secondary infections.

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