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# Paper-based enzyme-free immunoassay for rapid detection and subtyping of influenza A H1N1 and H3N2 viruses

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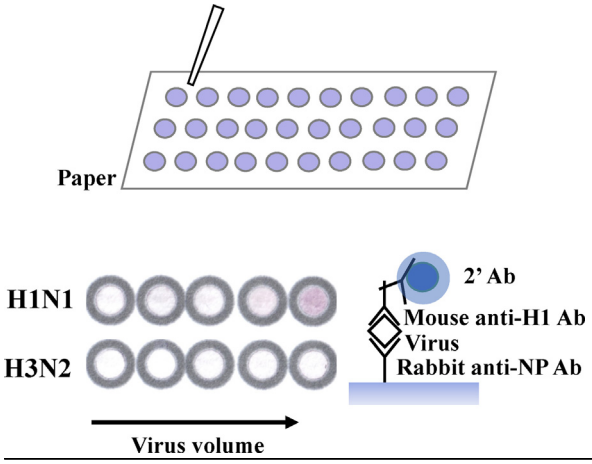
## HIGHLIGHTS

- Development of a paper-based enzyme-free sandwich immunoassay.
- Optimization of the conditions of paper-based immunoassay.
- Detecting and subtyping of influenza viruses with the detection limits of $2.7 \times 10^3$ pfu/assay for H1 detection and $2.7 \times 10^4$ pfu/assay for H3 detection.
- Demonstration of influenza screening with less sample and reagent volume (5 μL) in shorter period of time (around 1 h).
- Evaluation of the paper-based immunoassay using infected cell lysate and clinical samples.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Development of rapid screening in the ambulatory environment is the most pressing needs for the control of spread of infectious disease. Despite there are many methods to detect the immunoassay results, quantitative measurement in rapid disease screening is still a great challenge for point-of-care applications. In this work, based on the internal structural protein, i.e., nucleoprotein (NP), and outer surface glycoproteins, i.e., H1 and H3, of the influenza viruses, specific and sensitive immunoassay on paper-based platform was evaluated and confirmed. Detection and subtyping of influenza A H1N1 and H3N2 viruses found in people were demonstrated by colorimetric paper-based sandwich immunoassay. Concentration-dependent response to influenza viruses was shown and the detection limits could

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achieve $2.7 \times 10^3$ pfu/assay for H1 detection and $2.7 \times 10^4$ pfu/assay for H3 detection, which are within the clinical relevant level. Moreover, detection of influenza virus from infected cell lysate and clinical samples was demonstrated to further confirm the reliability of the paper-based immunoassay. The use of paper for the development of diagnostic devices has the advantages of lightweight, ease-of-use, and low cost and paper-based immunoassay is appropriate to apply for rapid screening in point-of-care applications.



## 1. Introduction

In the past century, several major breakouts of pandemic have killed millions of people worldwide. In 1918–1919, Spanish flu pandemic (influenza A virus of H1N1 subtype) caused the deaths of 50 million to 100 million people worldwide [1]. In 1968–1969, Hong Kong flu pandemic (influenza A virus of H3N2 subtype) killed one million people worldwide [2]. Most recently, a new H1N1 virus recognized as a swine-origin influenza virus with a new combination of genes from American pigs, Eurasian pigs, birds, and humans infected people in 2009 and led to pandemics [3]. More importantly, 250,000–500,000 people annually and up to million people in some pandemic years passed away could be attributed to the spread of influenza viruses. The spread of influenza viruses has become a global health concern and efficient diagnosis is very important for the control of spread of influenza viruses.

Influenza viruses are categorized into three types, i.e., influenza A, B, and C [4]. Influenza A and B viruses cause seasonal epidemics and respiratory illness in humans and can potentially cause pandemic outbreak. Infection of influenza C virus may only result in minor symptoms and rarely causes epidemics. Further subtyping of influenza A virus is based on the antigenic properties of the outer surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) given as HxNy subtype. According to the Centers for Diseases Control and Prevention, USA, there are 18 different hemagglutinin subtypes (H1–H18) and 11 different neuraminidase subtypes (N1–N11) in different combinations from birds, animals, and human beings. In recent years, H1N1 and H3N2 of influenza A viruses are the major subtypes circulating in humans.

Traditionally, enzyme-linked immunosorbent assay (ELISA) is one of the widely used and standard diagnostic techniques. This bio-analytical technique is to measure the presence and concentration of antibody or antigen in biological liquid. It is based on the specific interaction between an antibody and its antigen. Hence, antigen of a particular disease can be detected by the according known antibody. A high specificity of the antibody and antigen pair is selected in the ELISA kits for diagnostic applications. In the screening of influenza infections, based on the recognition of influenza virus antigens, ELISA can be used for detecting and subtyping of influenza infections. However, the entire process is operated by dedicated equipment in a well-equipped laboratory, that makes diagnostic service in hospital expensive and time-consuming. Therefore, the need of rapid screening for quantitative measurement that can operate onsite is emphasized recently. Rapid screening for point-of-care (POC) applications often refers to portable, fast response, simple operation, and inexpensive device involved [5]. Such technologies hold great impact on improving global health [6,7].

In the past decade, microfluidic systems have been extensively developed for various biomedical applications and a lot of excellent demonstrations have been reported [8,9]. Detection of influenza viruses has also demonstrated based on microfluidic technology [10–12]. Although these systems are much more simplified than traditional bio-analytical instruments, but they are still not readily accessible to untrained personnel and not appropriate for POC applications [13]. Therefore, paper-based microfluidics has been recently proposed for a new class of POC diagnostic device [14]. A number of biomedical analyses have been demonstrated including colorimetric bio-assays [14–16], electrochemical bio-assays [17–19], and paper-based ELISA [20–22]. The use of paper for the development of diagnostic devices has the advantages of lightweight, ease-of-use, and low cost and paper-based microfluidic devices are appropriate to apply for rapid screening in POC applications. For example, paper-based ELISA can be completed within an hour, whereas conventional ELISA requires at least 6 h to complete. Moreover, the usage of samples and reagents is significantly reduced in paper-based ELISA, since only 3 μL is required for each test zone. That means great reduction of diagnostic cost compared with conventional ELISA. Also, the ease of fabrication of paper microzone plates also opens opportunities for a wide range of non-standard formats for customized assays [20]. To date, paper-based ELISA has been demonstrated to detect HIV-1 envelope antigen gp41 [20], human performance biomarker neuropeptide Y [21], dengue virus serotype-2 nonstructural protein-1 antigen [22], and tumor markers [23]. Most of the aforementioned works were based on indirect immunoassay model [20–22]. In this work, the highly sensitive and specific sandwich immunoassay model was applied to demonstrate the rapid detection and subtyping of influenza A H1N1 and H3N2 viruses.

In this work, detection and subtyping of influenza A H1N1 and H3N2 viruses found in people were demonstrated by colorimetric paper-based immunoassay. The paper microzone plates with $8 \times 6$ array of circular test zones for running multiple immunoassays in parallel were fabricated by wax-printing [24]. Each test zone was 6 mm in diameter and required 5 μL of solution to completely wet the test zone. We also examined different colorimetric substrates, immobilization strength of antibody, and blocking conditions in order to optimize the conditions used in the paper-based immunoassay. For the development of sandwich immunoassay model, internal structural protein, i.e., nucleoprotein (NP), of influenza virus was selected to be captured for the detection of influenza A viruses [25,26]. Outer surface glycoproteins, i.e., H1 and H3, of influenza virus were used to subtype the influenza A H1N1 and H3N2 viruses. Specificity and sensitivity were respectively evaluated and confirmed on the paper-based platform. The results from this study showed that the influenza screening can be quantitatively performed with less sample and reagent volume (5 μL) in shorter period of time (around 1 h). Compared with the commercial kit used for rapid diagnostic testing (QuickVue Influenza A + B), it can only differentiate influenza A and B viruses by NP protein and cannot do subtyping of influenza A H1N1 and H3N2 viruses. More importantly, it was reported that the sensitivity of this kit is poor [27,28]. Therefore, the most reliable diagnostic of influenza virus is based on real-time reverse-transcriptase polymerase-chain-reaction (RT-PCR) currently. The RT-PCR normally takes 6 h to be completed and requires to be operated in a high standard laboratory. Such that, the paper-based immunoassay provides rapid detection and subtyping of influenza A H1N1 and H3N2 viruses within 1 h. Also, the operation requirement of