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A new point-of-care test for the diagnosis of infectious diseases based on multiplex lateral flow immunoassays

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Infectious diseases are transmissible or communicable illnesses and can spread quickly in some areas and become epidemics. It is critical to quickly diagnose initial infections and prevent further spread through *in vitro* diagnosis. However, current detection strategies have exhibited a lack of balance with regard to accuracy, time consumption, and portability until recently (*e.g.* serology, culturing, molecular tests, *etc.*). Alternatively, many studies have focused on point-of-care testing (POCT), which combines simple, rapid, and exact on-site diagnostic platforms. Moreover, multiplex detectability is necessary for emergency treatment depending on the stage of the disease or interactional infections. The lateral flow assay (LFA) is the most popular diagnostic tool that meets the required standards for colorimetric assays. Here, we review lateral flow assays based on the immune reactions for the simultaneous diagnosis of infectious diseases as the POC test. The assays employed various forms and approaches in terms of the multiplexing level system for improving the sensitivity and specificity. We briefly describe the state-of-the-art infection diagnostic methods and published performances that have been classified into three categories based on the application forms of the lateral flow immunoassay. Also, we discuss further uses of LFA and other technologies for more effective infectious disease POCT.

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Introduction

Infectious diseases are mainly caused by pathogenic microorganisms such as bacteria, viruses, parasites, or fungi that can be spread, directly or indirectly, from one person to another.[1] Diagnostic tests play an important role in the medical care of patients with suspected infections. Traditional detection methods for specific pathogens include Gram-stains, pathogen culturing, and biochemical methods as well as serology (*e.g.*, antibody detection through enzyme immunoassays (EIA) and enzyme-linked immunosorbent assays) and molecular tests (polymerase chain reaction (PCR) and other nucleic acid–based amplification technologies (NAATs)).[2,3] These techniques have significantly contributed to the diagnosis, prevention, and treatment of various infectious diseases. However, they have also exhibited many limitations, such as being time consuming, expensive, requiring complex analytical instruments, and requiring skilled technicians.[4] In particular, in developing countries, these techniques have further limitations because of limited resources and specialized personnel.[4] For example, in 2015, over 85% of the 5.7 million global deaths caused by infectious and parasitic diseases occurred in low and lower-middle income settings.[5] Therefore, point-of-care testing (POCT), which is a simple, rapid, inexpensive, and highly accurate on-site diagnostic platform, needs to be developed for the prevention and therapy of transmissible diseases. In this regard, POCT for diseases represents the most promising and rapidly growing market within the *in vitro* diagnostics (IVD) industry. The infectious disease POCT market is expected to reach over USD 1.17 billion by 2022, representing a compound annual growth rate of 7.7%.[6] A number of factors including the growing popularity of infectious diseases in developing countries, the rising usage of home-based POC devices, the expanding test menu, and technological advancements in molecular testing and lab-on-chip technology are stimulating the demand for infectious disease POCT (Table 1).[7] As the demand for infectious disease POCT increases, manufacturing companies compete to develop next-generation POC platforms that are faster, easier to operate, and more reliable. In 2004, the World Health Organization (WHO) introduced a

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Table 1 Drivers of and barriers to growth in the infectious disease POCT market

Driver	Barrier
Increasing prevalence of infectious diseases	
Fast turnaround time (TAT)	
Emergence of more accurate molecular point of care (POC) technologies	
Advancing POC technology and the expansion of the test menu	Complexity and added cost of management of POCT devices and tests, limiting market growth potential
Growing home-testing market to encourage further adoption of POC devices	Competition with centralized labs and hospital stat labs, reducing the rate of POCT adoption
CLIA-waived status for increasing the number of infectious disease tests, enabling their usage by healthcare providers in non-traditional laboratory settings, including offices of physicians	
The industry's continued push for a treatment paradigm shift to value-based and patient-centered care	

set of seven criteria that a POC diagnostic test should ideally meet in order to be useful in a resource-limited setting.[8] These guidelines are known as the ASSURED criteria which state that a device should be affordable, sensitive and specific (few false-negatives and false-positives, respectively), user-friendly, rapid and robust, equipment-free (*e.g.*, does not require refrigeration), and easily delivered (accessible to end users).[8] For example, the lateral flow immunoassay (LFIA) platform is known as an ideal diagnostic test with features including less time consumption, easy operation, durable stability, low cost for POC tests in limited-resource settings and emergency departments.[9] Also, LFIA tests are able to show the existence of pathogens in various biological samples (*e.g.* whole blood, plasma, serum, saliva, *etc*.) and the results can be confirmed with the naked eye.[10,11] Recently, many studies have applied the multiplex biomarker detection format to LFIA for rapid recognition of several target biomolecules in a single platform. The important milestones of the multiplex detection format are its capabilities of rapid analysis (because multiple analytes must be analyzed at the same time) at different stages of disease, detection of co-infections at once, reducing test costs per assay (no need to perform the test separately), and examining other inter-dependent situations.[12–16] Furthermore, it allows the identification of a subclinically infected individual through targeting of several biomarkers and leads the way for highly sensitive and optimized POCT.[17] Nonetheless, several clinical evaluations have shown that many platforms do not meet the required criteria for practical use.[18] The main barriers have been their relatively low accuracy and limited sensitivity to be applied as infectious disease POC tests based on colorimetric assays. Another concern is related to the probable cross-reactivity that limits the number and types of biomarkers for multiplexing. For overcoming the limits, two factors are commonly examined: the use of differentiated detection labels and the development of optical strip readers which improve the fine detectability as ultra-sensitive multiplex LFA sensors.[19]

This review focused on recent POC tests based on multiplex lateral flow immunoassays in the context of infectious diseases with similar symptoms such as foodborne diseases, respiratory tract infections (RTIs), and emerging infectious diseases (*e.g.*, Ebola virus). We aimed to summarize the information about recently approved and commercially available products and state-of-the-art development through a systematic literature review. To this end, this paper classified multiplex LFIA into three categories according to the application: (1) the detection of several analytes on a single strip, (2) multi-detection with multiple strips, and (3) integration of lateral flow and microassay technologies (Fig. 1). We also briefly discussed the current implementation challenges and future perspectives with practical concerns.

Multiplexing lateral immunoassay biosensors

Lateral flow immunoassays (LFIA) are based on the inducement of a sample stream, containing the target analytes, along a strip.[10] The strip basically consists of a membrane and some functional pads including a sample pad, conjugate pad, and adsorbent pad on a strip. The nano-sized particles are immobilized on the conjugated pad to bind and indicate that the target molecules captured the antibody on the surface of a

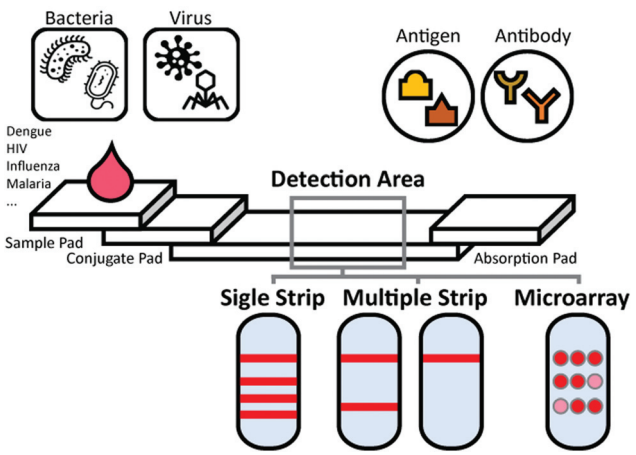

Fig. 1 Schematic of multiplex lateral flow immunoassay targeting infectious disease causing viral agents.

Table 2 List of multiplexed lateral flow immunoassays for POCT of infectious diseases

	Purpose	Target	Ref.
Multiplexing on a single strip	Two-color LFAs for the multiplex IgG/IgM detection of several febrile illnesses	Dengue virus (IgG/IgM) Chikungunya virus (IgG/IgM)	20
	Multiplex detection of humoral immune responses to infectious disease pathogens	Antibodies against HIV-1 and -2, *Myobacterium tuberculosis*, and Hepatitis C virus in plasma	21
	Three-colored multiplexed lateral flow POC sensor for the simultaneous diagnosis of severe acute febrile illnesses	Dengue virus NS1 protein, Yellow Fever Virus NS1 protein, and Ebola virus, Zaire strain glycoprotein GP	22
	Fast diagnosis of Scrub typhus in many endemic regions	IgG and IgM antibodies to *O. tsutsugamushi*	23
	Stable multiplex LFA using AMP for the simultaneous detection and identification of Shiga toxin-producing *Escherichia coli* (STEC) serogroup	STEC O157, O26, and O111	24
	Multiplex diagnosis of HIV and HCV to identify the co-infected individuals	Anti-HIV IgG, Anti-HCV IgG, Anti-HAV IgG and IgM	25
Multiplexing with multiple strips	Both detection and differentiation of influenza A and B type viruses in respiratory samples	Influenza A and B virus antigen	26
	Multiplex immuno-disc sensor for cystic fibrosis caused by bacterial infection	*Pseudomonas aeruginosa*, *Staphylococcus aureus*	27
	Rapid, simultaneous detection of 10 epidemic foodborne pathogens for food safety and low-occurrence of foodborne disease	*E. coli* O157:H7, *S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. typhi, S. enteritidis*, *S. choleraesuis*, *V. cholera* O1, *V. cholera* O139, and *V. parahaemolyticus*	28
Integration of lateral flow and microarray technologies	Mosquito-control measures in many endemic areas	Malaria antigen *Plasmodium falciparum* histidine-rich protein 2	29

nitrocellulose membrane. The appearance of a line of conjugated reporter particles represents a positive result. There have been academic intentions to detect multiplex pathogens on the LFIA system for POCT through the development of special conjugated particles for accurate results and originality as a diagnostic kit. Early consideration must be given on whether to attempt to capture analytes and obtain results on a strip or generate numerous strips within a single cartridge. We present comprehensive descriptions of these multiplexing strategies and published performances in Table 2.

The detection of several analytes on a single lateral flow immunoassay strip

In the LFIA platform, multiplexing assay on a single strip has previously been achieved through the introduction of additional detection antibodies of the same label and through the addition of associated test lines that are spatially separated from each other.[20] The tests typically involve performing an antigen-specific test to detect the presence of pathogens (*e.g.*, non-structural protein 1 of the dengue virus and protein E2 of the Chikungunya virus), which are present at high concentrations in human blood during the early clinical stage of infection.[20] For example, Corstjens *et al.* reported on a rapid assay platform for the multiplex detection of antibodies against Human Immunodeficiency Virus, Hepatitis C, and *Mycobacterium tuberculosis* (HIV, HCV, and TB) which captured distinct test lines on a single membrane.[21] The antibodies to these infectious disease pathogens were captured at the corresponding test lines which immobilized target-specific antigens and the remaining IgGs conjugated to an anti-human IgG on the control line. Subsequently, some pioneering studies have reported the detection of several infectious agents indicated by distinct colors on a single LFIA. Yen *et al.* distinguished multiple pathogens (*e.g.*, dengue, yellow fever, and Ebola virus) with three distinct labels using multi-colored silver nanoparticles as indicators on a strip (Fig. 2A).[22] The limits of detection (LODs) for the three viral proteins were all in the range of 150 ng mL^{-1}. The authors also detected multiple pathogens mixture on individual colored test lines corresponding to the antigens. In addition, all the target proteins that were captured on the single test line were distinguished by the RGB analysis of labelled color silver nanoparticles differentiated among target molecules. The evident signal and scant non-specific binding were demonstrated through both scenarios of each of the test line or a single capture area. As a result, these results can facilitate miniaturization through a number of analyte detection processes in a few test lines.

Serological tests carry out supporting result of antigen-specific test since infectious agent concentrations fall below a detectable range in the later stages of infection that make diagnose difficultly.[20] The tests mainly rely on detecting Immunoglobulin M (IgM) antibodies and are supplemented by concentration of Immunoglobulin G (IgG) antibodies for diagnosis of acute infectious diseases. Therefore, it is vital to distinguish IgG and IgM for determining the state of the disease. For these tests, a single Rapid Diagnostic Test (RDT) strip is the current state-of-the-art tool for duplex immunoglobulin detection with two capture lines (one for IgG and the other for IgM detection). For instance, Wilkinson *et al.* recommended the rapid lateral flow assay for the diagnosis of Scrub typhus with IgG and IgM of *Orientia tsutsugamushi* that causes the disease.[23] Gold nanoparticles are conjugated with truncated

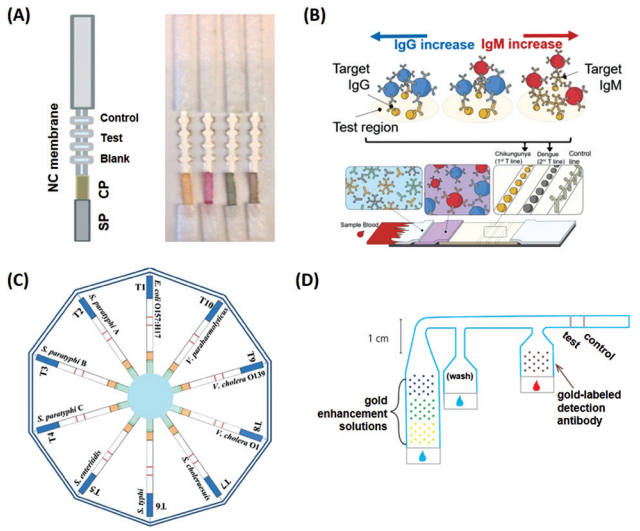

Fig. 2 Schemes of multiplexing lateral flow immunoassay for simultaneous detection of (A) YFV, ZEBOV and DENV with three colored nanoparticles on a single strip. (Reproduced from ref. 18 with permission from The Royal Society of Chemistry.) (B) anti-CHIKV and anti-DENV IgG/IgM with a two-color latex probe on a single strip. (Reprinted with permission from S. Lee, *et al.*, *Anal Chem.*, 2016, **88**(17), 8359–8363. Copyright 2016 American Chemical Society.) (C) 10 epidemic foodborne pathogens on a disc with multiple strips. (Reprinted from Y. Zhao *et al.*, *Sci. Rep.*, 2016, **6**, 21342 under open access license CC BY-NC, Copyright 2016 Springer Nature.) (D) malaria pathogen with integration of micro-arrays. (Reprinted from G. E. Fridley *et al.*, *Anal. Chem.*, 2014, **86**, 6447–6453 under open access license CC BY-NC, Copyright 2014 American Chemical Society.).

recombinant protein (r56) which originates from the 56 kDa major outer membrane protein of *O. tsutsugamushi* and combines with IgG and/or IgM antibodies to the pathogen on each test line after 10 min of conjugation. The LFIA discriminated the two types of antibodies from the patient's serum and indicated higher sensitivity than the commercial analytical method for r56. Unfortunately, it had cross-reactivity against hepatitis A, early EBV infection, and malaria. Similar to assays based on groups of immunoglobulin, other studies from Yonekita's group distinguished three serogroups of Shiga toxin-producing *Escherichia coli* (STEC O157, O26, and O111) visualized as reddish-purple lines on a strip.[24] The study demonstrated stable multiplex LFIA using antimicrobial peptides (AMPs) for the simultaneous detection and identification of the serogroups within 15 minutes. Biotin labelled AMP probes, which bind to STEC, conjugate with streptavidin-colloidal gold and the complexes successfully indicate the existence of analytes from liquid and food samples (*e.g.*, 25 g of ground beef after 18 h of enrichment). The proposed assay showed an LOD of 10^4 CFU mL^{-1} and the non-reaction to other serogroups or non-*E. coli*.

In such cases, the limitations of the strip are associated with the verification of only a single infectious disease, and would increase the use of other pathogen-specific strips in parallel for the detection of immunoglobulins to other pathogens. Recently, Lee *et al.* reported on a two-color latex probed LFIA that enables the 4-plex detection of IgG/IgM to two distinct acute undifferentiated febrile illnesses (AFIs), Chikungunya virus (CHIKV) and dengue virus (DENV), on a single strip (Fig. 2B).[20] First of all, the distinct locations of the test region allow for the differentiation of diseases such as Chikungunya or dengue. When the sample contains pathogen-specific immunoglobulins or their two types, POCT exhibits blue, red, or mixed colors developed on the test regions which could be used to correctly identify 12 different diagnostic scenarios involving anti-CHIKV and anti-DENV IgG/IgM. In a large number of cases, typical antigenic peptide or protein probes generate irregular locations and aggregated clusters on performing the assay that obstruct the binding of target biomolecules to the probes. To overcome this drawback of conventional LFIAs, Lee's group suggested lateral flow assays based on engineered 'protein-ticles' for the simultaneous detection of several infectious diseases including AIDS, Hepatitis C, and Hepatitis A within a half hour.[25] The particles are formed through the self-assembly of protein monomers or constituents, and distinct viral antigens corresponding to the disease are linked on its C-terminus as probes to capture antibodies against each infectious agent. The 3D probes are capable of being well-oriented, stable, and binding effectively to target molecules on the test line of the assay. The authors demonstrated the detection of anti-HIV IgG, anti-HCV IgG, and anti-HAV IgG and IgM from individual- or co-infected patient sera. These viral diseases were diagnosed with 100% sensitivity with 64-fold sample dilution and accurately reported through duplex or triplex tests on a single assay. The magnificent LFIA system for viral antibody detection enabled the application of intractable multiplex diagnoses of infectious diseases through engineered 3D proteinticle probes.

Multi-detection with multiplex lateral flow immunoassay strips

However, multiplexing several test lines on a single LFIA strip is confined to physical limits, the number of conjugates, and the flow changes by passing on multiple lines.[30] Furthermore, the assay cut-off threshold value is modified since the result relies on the flow distance of the test line from the sample pad.[21] Physical limitations may be avoided using recently suggested creative designs with multiple strips and spot arraying on the test area of the membrane through a single sample application.[30] Cazacu *et al.* proposed a new LFIA (Xpect Flu A/B; Remel Inc., Lenexa, Kansas) to simultaneously detect and distinguish influenza A and B type viruses from 400 respiratory secretions on two separate test strips of a single cartridge.[26] They referenced the standard of viral cultures in specimens collected from patients who presented with respiratory infections or flu-like symptoms. Consequently, the rapid test resulted in great performance factors such as 94.4% sensitivity and 100% specificity within 15 min of the test. Moreover, recent studies have suggested various forms of multiplex lateral flow immunoassay platforms such as a fork, peace symbol, and disc for the diagnosis of other infectious diseases on pathogen-specific strips. Li's group demonstrated three strips of immuno-disc sensors for the multiplex detection of

bacterial infection caused cystic fibrosis (CF).[27] The sensor detects the presence of bacterial pathogens (*e.g. Pseudomonas aeruginosa and Staphylococcus aureus*) after 5 min of dropping the sample onto the centre of discs and their concentrations from 0.5×10^3 to 5×10^3 CFU mL^{-1} through the color intensity of the test line with high specificity. They additionally developed a color intensity identifier device for a reliable and compact POCT system. Zhao *et al.* proposed a 10-channel lateral flow assay for the rapid and simultaneous detection of 10 epidemic-causing foodborne pathogens such as *E. coli* O157:H7, *S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. typhi*, *S. enteritidis*, *S. choleraesuis*, *V. cholera* O1, *V. cholera* O139, and *V. parahaemolyticus* (Fig. 2C).[28] The assay consists of 10 single-target strips integrated into one disc and only the target bacterium presented a strong signal in the corresponding detection channel within 20 minutes. Simultaneous multiplex detection was also highly correctively quantified with the correlative calibration curve as 10^7 CFU mL^{-1}, but only four of the ten samples were accurately classified on all 10 target bacteria contaminated food tests.

Integration of lateral flow and micro-assay technologies

While the abovementioned studies have reported magnificent LFIAs for the simultaneous diagnosis of several infectious diseases and application of POC tests, they have continued to use simplex or low-plex lateral flow immunoassays with two to ten analyte detection processes and may suffer limited quantitative ability and diagnostic validity.[31] Therefore, some groups have suggested combining a lateral flow assay framework with protein microarray patterning for the rapid and accurate detection of single viral infectious agents (Fig. 2D).[29,32] The protein microarrays are extensively utilized tools for diagnostic biomarker analysis in the cases of infection, allergy, and instant autoimmunity.[33] Since all of the single spots concurrently appear on a microarray and are given the exact same volume from the flow of the sample, the assay is capable of exactly detecting the number of targets with as many microspots and reduce the potential variability that occurs with the aliquot preparations.[34] Recent studies have reported high-throughput of decades to hundreds-plexed lateral flow protein microarray devices based on antigen-specific antibody responses with sensitive and rapid analytical assays.[34–36] Furthermore, a company (Zyomyx, Hayward, CA) has manufactured high-density arrays using photolithography to enable the detection of up to 10 000 proteins in parallel.[37] Although such novel microarrays have been verifiably profitable for the simultaneous detection of pathogens, expensive instruments and skilled personnel are still needed to fabricate the array platforms which are unsuitable in endemic areas with destitute settings. In addition, lateral flow and micro-assay integrated technologies have yet to improve in their discrimination among other infectious diseases with similar conditions for biological analysis and POC application.[38] Due to the aforementioned drawbacks of the microarray, integrated lateral flow immunoassays will require further studies and evaluations to meet the ASSURED criteria for POCT of infectious diseases.[31] Therefore, further improvements are required to move forward with ideal high-technology in the medical diagnostic field covering the broad ranges of disease stages and numerous statuses of viral agents, immunoglobulins, and biomarkers.

Conclusions

To date, numerous efforts have been made to develop lateral flow assays for the optimal POCT of infectious diseases in resource-limited settings. Some studies have suggested simultaneous detection processes with several strips corresponding to the pathogens, while other groups have proposed multiplex infection detection on a single strip. Recently, the integration of LFIA and microarrays has been demonstrated to be able to diagnose many infectious diseases based on a number of biomarkers with accurate and rapid assays. The state-of-art promising multiplex lateral flow assays have been consisted of remarkable indicators, which are formed various materials and morphological properties, for sensitivity enhancement and high potential as POC tests. Beside colloidal gold nanoparticles, novel labels have recently been introduced for immunoassay such as super-paramagnetic nanobeads,[39,40] copper,[41] and fluorescent conjugates.[42,43] Also, assay reader devices have been developed to resolve the limits of former LFIA systems and offer prospective functions (*e.g.* cameras, spectrometers, and magneto-inductive sensors).[44,45] On the other hand, their performances have yet to be sufficient enough for use in settings of developed countries. To this end, we suggest that improvements to the next steps should be considered to prove the stability of the assays, ease of fabrication of the platforms, and practicality in emergency use. In addition, such convergence of biotechnology and mobile devices could lead to breakthroughs related to rapid and accurate clinical analysis. For example, up-to-date smartphone coupled diagnostics is able to quantify a biomarker below 15 μg L^{-1} from a drop of body fluid and manage the assessment globally only with an analyzed app and a disposable LFIA strip.[46] This simple assay is eligible to be a more reliable and high potential diagnostic method in the POCT field.

Conflicts of interest

No potential conflicts of interest relevant to this article were reported.

Acknowledgements

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