Article

Discovery Medicine 2026; 38(210): 2190–2200
https://doi.org/10.24976/Discov.Med.202638210.171

Development and Evaluation of a Multiplex Lateral Flow Immunoassay for Multiple Respiratory Virus Detection

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Submitted: 10 February 2026 Revised: 1 June 2026 Accepted: 10 June 2026 Published: 23 July 2026

Background: Simultaneous detection of influenza A virus (IAV), influenza B virus (IBV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is essential for rapid differential diagnosis in febrile patients, particularly in the context of co-circulating respiratory viruses. However, conventional gold nanoparticle-based lateral flow immunoassays are limited by single-color readouts and insufficient sensitivity, restricting their multiplex detection capability. This study aimed to develop a multicolor latex-based immunochromatographic strip as a rapid, sensitive, and reliable tool for analyte detection.

Methods: In this study, a multicolor latex microsphere-based immunochromatographic assay was developed for the rapid and simultaneous detection of SARS-CoV-2, IAV, and IBV antigens. Colored latex microspheres conjugated with virus-specific antibodies enabled visual differentiation of targets (SARS-CoV-2: red; IAV: blue; IBV: black). The analytical and clinical performance of the assay were systematically evaluated.

Results: The assay enabled detection within 15 minutes, with a limit of detection (LOD) of 10^2 $TCID_{50}$/mL for all three viruses. Clinical evaluation showed a sensitivity of 97.73% (215/220) and specificity of 100% (200/200) for SARS-CoV-2 detection, with an overall concordance rate of 98.81% (415/420) and a Kappa value of 0.9762 ($p < 0.05$), indicating excellent agreement with reference methods. Detection consistency for IAV and IBV was 100%. No cross-reactivity was observed with five common respiratory pathogens.

Conclusions: This multicolor latex-based immunochromatographic strip exhibits rapid, sensitive, and reliable performance for analyte detection without requiring specialized training. It shows strong potential for home self-testing and early screening in settings where SARS-CoV-2 and influenza co-circulate.

Keywords: SARS-CoV-2 antigen; influenza A virus antigen; influenza B virus antigen; colored latex; lateral flow immunoassay; methodological evaluation

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel human pathogen identified in late 2019 [1], triggered the global COVID-19 pandemic. As the global seasonal influenza transmission period approaches, populations face the dual threat of influenza viruses and SARS-CoV-2. Among these, Influenza A virus (IAV) and Influenza B virus (IBV) are of primary public health concern [2]. Statistics indicate that influenza causes approximately 3–5 million severe cases and 294,000–518,000 deaths globally each year. Furthermore, studies have reported coinfection cases, with patients infected by both SARS-CoV-2 and influenza facing a significantly higher risk of mortality [3,4].

Early symptoms of COVID-19, such as fever, dry cough, and fatigue, are non-specific. Some patients also exhibit upper respiratory symptoms like nasal congestion, rhinorrhea, and sore throat. Clinically, mild to moderate COVID-19 is difficult to distinguish from IAV and IBV infections based solely on symptoms. In the context of the ongoing pandemic, this similarity poses diagnostic challenges for physicians and exacerbates patient anxiety. Therefore, a diagnostic kit capable of simultaneously detecting and differentiating SARS-CoV-2, IAV, and IBV is urgently needed for rapid triage.

Currently, real-time quantitative polymerase chain reaction (RT-qPCR) is the recommended gold standard for COVID-19 diagnosis [5,6]. However, RT-qPCR requires specialized equipment and trained personnel, is time-consuming, and costly, limiting its utility for rapid identification and transmission chain disruption [5,7]. In contrast, antigen detection is convenient, rapid, and resource-efficient. It facilitates early triage and management of sus-

pected cases and meets the demands of large-scale screening, thereby complementing nucleic acid testing. Moreover, antigen testing imposes less stringent requirements on sample preservation compared to molecular methods [8,9,10].

Rapid antigen detection based on the double-antibody sandwich method improves specificity and reduces false positives. While current commercial reagents generally exhibit high specificity, their sensitivity varies significantly (34%–86%) [11]. Additionally, most combined test strips utilize single-color tracers (e.g., colloidal gold), and rely solely on the position of the test line for identification, which can lead to misinterpretation.

In this study, we developed a rapid detection kit for SARS-CoV-2 N protein, IAV, and IBV antigens using a multicolor latex tracer-based immunochromatographic sandwich assay. This method enables the distinct identification of three viruses on a single strip, with naked-eye interpretation possible within 15 minutes, making it suitable for home use.

Methods

Materials

Colored latex microspheres were supplied by DIAN BioTec Co., Ltd. (Hangzhou, China). Bovine serum albumin (BSA) was purchased from Equitech-Bio, Inc. (Kerrville, TX, USA; Cat. No. BAC61). Trehalose, sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, and potassium chloride (analytical reagent grade) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). (3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, ≥98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA; Cat. No. E6383). Nitrocellulose membranes and glass fiber membranes were obtained from Merck Millipore Ltd. (Burlington, MA, USA). Nitrocellulose membranes (Hi-Flow™ Plus Membrane 135) and glass fiber membranes (GFCP203000) were obtained from Merck Millipore Ltd. (Burlington, MA, USA). Absorbent pads (high-capacity cellulose-based absorbent material) and polyvinyl chloride (PVC) backing cards (adhesive-coated rigid support sheets for lateral flow assembly) were obtained from Jieyi Biotechnology Co., Ltd. (Shanghai, China). Mouse anti-SARS-CoV-2 nucleocapsid (N) protein monoclonal antibody pairs were obtained from HyTest Ltd. (Turku, Finland, Cat. No. 3CV4). These antibodies were supplied as validated matched pairs for sandwich immunoassay development. Mouse monoclonal antibodies against influenza A virus (IAV) and influenza B virus (IBV) were obtained from Xiamen Tongrenxin Biotech Co., Ltd. (Xiamen, Fujian, China, Cat No. FLU21, FLU23), and were supplied as validated antibody pairs for immunochromatographic assay development. Goat anti-mouse IgG was obtained from Hangzhou Starter Biotechnology Co., Ltd. (Hangzhou, China, Cat No. S0B4006) and used as the control line capture antibody in the lateral flow assay. Heat-inactivated SARS-CoV-2, IAV, and IBV viral materials were purchased from Baikang Biotech Co., Ltd. (Hangzhou, China) and were consistently used across all analytical performance evaluations, including limit of detection (LOD) assessment and concentration-dependent detection experiments. All samples were collected as part of routine clinical workflows and uniformly stored at –80 °C prior to analysis to ensure consistency and sample integrity. The SARS-CoV-2 sample source with 220 positive cases and 200 nucleic acid-negative cases was clinically evaluated in the DIAN Diagnostics Group Co., Ltd.

The SARS-CoV-2 nucleic acid detection was performed using a Cobas Z480 Analyzer (Roche Diagnostics GmbH, Mannheim, Baden-Württemberg, Germany), and the antigen detection kit developed in this study was used in parallel for comparative testing using nasal swab samples. For IAV and IBV, clinical samples included 231 cases positive for IAV antigen but negative for IBV antigen, 272 cases positive for IBV antigen but negative for IAV antigen, and 200 cases negative for both IAV and IBV. These samples were evaluated using the multicolor latex-based antigen detection kit developed in this study and Alere BinaxNOW™ Influenza A and B Test Kits (Abbott Diagnostics Scarborough, Inc., Scarborough, ME, USA; Cat. No. BNX416000), applied simultaneously to nasal swab samples for comparative analysis. The reaction was performed at room temperature (RT, approximately 25 °C), and the pH was controlled using a MES buffer system (pH 6.1). This study was conducted in accordance with the Declaration of Helsinki. This ethics statement covers all enrolled populations. All samples (healthy controls and patient samples) were collected between 2024 and 2025 from the clinical laboratory of Tongxiang Zhouquan Town Central Health Center, and the study was approved by the Clinical Research Ethics Committee of the Clinical Laboratory of Tongxiang Zhouquan Town Central Health Center (Approval No. SBQLL-2025-475).

Preparation of the Conjugation of Monoclonal Antibodies With Latex Microspheres

Briefly, 1 mL of red latex was mixed with 19 mL of MES buffer (10 mM, pH 6.1). Anti-SARS-CoV-2 monoclonal antibody (1 mg) was added to the suspension and incubated for 15 min at RT with magnetic stirring. Conjugation was achieved via carbodiimide-mediated coupling using EDC (1.375 mg) for 60 min, a widely used method for covalent binding between carboxyl-functionalized particles and protein amine groups [12,13,14]. Unreacted sites were blocked with 0.4 mL of blocking buffer for 60 min. Free antibodies were removed by centrifugation at 23,000 ×g for 30 min. The pellet was resuspended in 12 mL of borate buffer (0.2 M, pH 8) containing 3% BSA (w/v) via

ultrasonication. The conjugates were stored at 2–8 °C in the dark. Blue latex-anti-IAV and black latex-anti-IBV conjugates were prepared using the same protocol.

Characterization of Colored Latex Microspheres

Transmission electron microscopy (TEM) imaging was performed using a JEM-2100F transmission electron microscope (JEOL Ltd., Tokyo, Japan). UV-visible absorption spectra were recorded using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). Hydrodynamic size distribution was measured using a Zetasizer Nano ZS90 (Malvern Panalytical Ltd., Malvern, UK). Virus-specific capture antibodies (1.0 mg/mL) and goat anti-mouse polyclonal antibodies (1.0 mg/mL) were dispensed onto the NC membrane, respectively, at a rate of 1 μL/cm to form detection lines (T line) and control lines (C line) and dried at 37 ℃ for 24 h. The conjugation pads were prepared by evenly coating the latex-antibody conjugates on the conjugated pad with the conjugate diluent solution and drying at 37 ℃ for 24 h. Then, the prepared NC membrane, conjugate pad, sample pad, and absorbent pad were assembled on the PVC back pad, and the structure diagram of the test strip is shown in Fig. 1. The assembled big card was cut into 3-mm strips with a strip cutter and sealed in an aluminum foil bag.

Test Procedure of the Multi-Color Latex Tracer Immunochromatographic Strip

Nasal swabs were collected by inserting the soft end of the swab approximately 1.5 cm into the nostril, rotating it gently against the nasal wall at least four times for 15 seconds, and repeating the process in the other nostril using the same swab [15]. The swab was inserted into an extraction tube containing approximately 350 μL of buffer and rolled 10 times. The swab tip was broken off at the breakpoint, and the tube was capped tightly. Three drops (~70–80 μL) of the treated sample were added to the sample well of the test cassette. Results were read visually after 15 minutes; results observed after 20 minutes were considered invalid. Quantitative analysis was performed by scanning the strips with a digital reader (Shenzhen Yuanqin Biotech Co., Ltd., Shenzhen, China) and analyzing grayscale intensity using ImageJ software (version 1.53, National Institutes of Health, Bethesda, MD, USA). The T/C ratio (the grayscale intensity of the T line/the grayscale intensity of the C line) was used for data processing.

Performance Evaluation of the Multi-Color Latex Tracer Immune-Chromatographic Strip

To determine the LOD of the assay, heat-inactivated SARS-CoV-2, IAV, and IBV, obtained from Baikang Biotech Co., Ltd. (Nanjing, China), were spiked into the negative specimen, serially diluted, and tested under identical experimental conditions. The initial concentrations were set at 10^6 $TCID_{50}$/mL for SARS-CoV-2 and 10^4 $TCID_{50}$/mL for IAV and IBV, respectively, followed by stepwise dilutions. These starting concentrations were selected based on preliminary optimization experiments and previously reported viral load ranges in clinical respiratory specimens, which indicated that different respiratory viruses may exhibit distinct viral kinetics and concentration distributions across infection stages [16,17,18]. Negative samples used as the matrix for LOD determination were nasal swab eluates collected from healthy volunteers and confirmed to be negative for SARS-CoV-2, IAV, and IBV by reference diagnostic methods. Heat-inactivated SARS-CoV-2, IAV, and IBV were serially diluted in this negative matrix, and each concentration was tested in 20 replicates under identical experimental conditions. The LOD was defined as the lowest concentration yielding a detection rate of ≥95% (≥19/20 positive results) [19]. Detailed LOD results, including concentration levels, number of replicates, and detection rates, are provided in **Supplementary Table 1**.

The cross-reactivity study was conducted to evaluate potential interference from pathogens that may cause symptoms similar to SARS-CoV-2, IAV, or IBV infections. To assess the analytical specificity of the assay, a panel of five common respiratory pathogens, respiratory syncytial virus (RSV), Mycoplasma pneumoniae, parainfluenza virus, adenovirus, and rhinovirus, was selected. All pathogen materials were obtained from Wuhan Huizao Biotechnology Co., Ltd. (Wuhan, China) and prepared according to the manufacturer's instructions before analysis. These pathogens were chosen based on their high prevalence in respiratory infections, frequent co-circulation with SARS-CoV-2 and influenza viruses, and their potential to cause overlapping clinical symptoms such as fever and respiratory distress [20,21]. Moreover, epidemiological studies have demonstrated that these pathogens constitute a major proportion of circulating respiratory viruses and contribute significantly to the complexity of respiratory infection diagnosis due to their co-circulation and similar clinical presentations [22,23]. In addition, this panel includes both viral pathogens and an atypical bacterial pathogen (Mycoplasma pneumoniae), thereby representing diverse biological characteristics that may contribute to potential cross-reactivity. This selection strategy ensured that assay specificity was evaluated against clinically relevant and representative interfering pathogens.

Performance Evaluation of the Multi-Color Latex Tracer Immune-Chromatographic Strips Using Clinical Samples

Clinical evaluation was performed at DIAN Diagnostic Group Co., Ltd. for SARS-CoV-2, and at Tongxiang Zhouquan Town Central Health Center for Influenza A and B. For each patient, two nasal swabs were collected. All swabs were randomly blinded, and the nasal swabs were tested using the Three Antigen Test Kit according to the

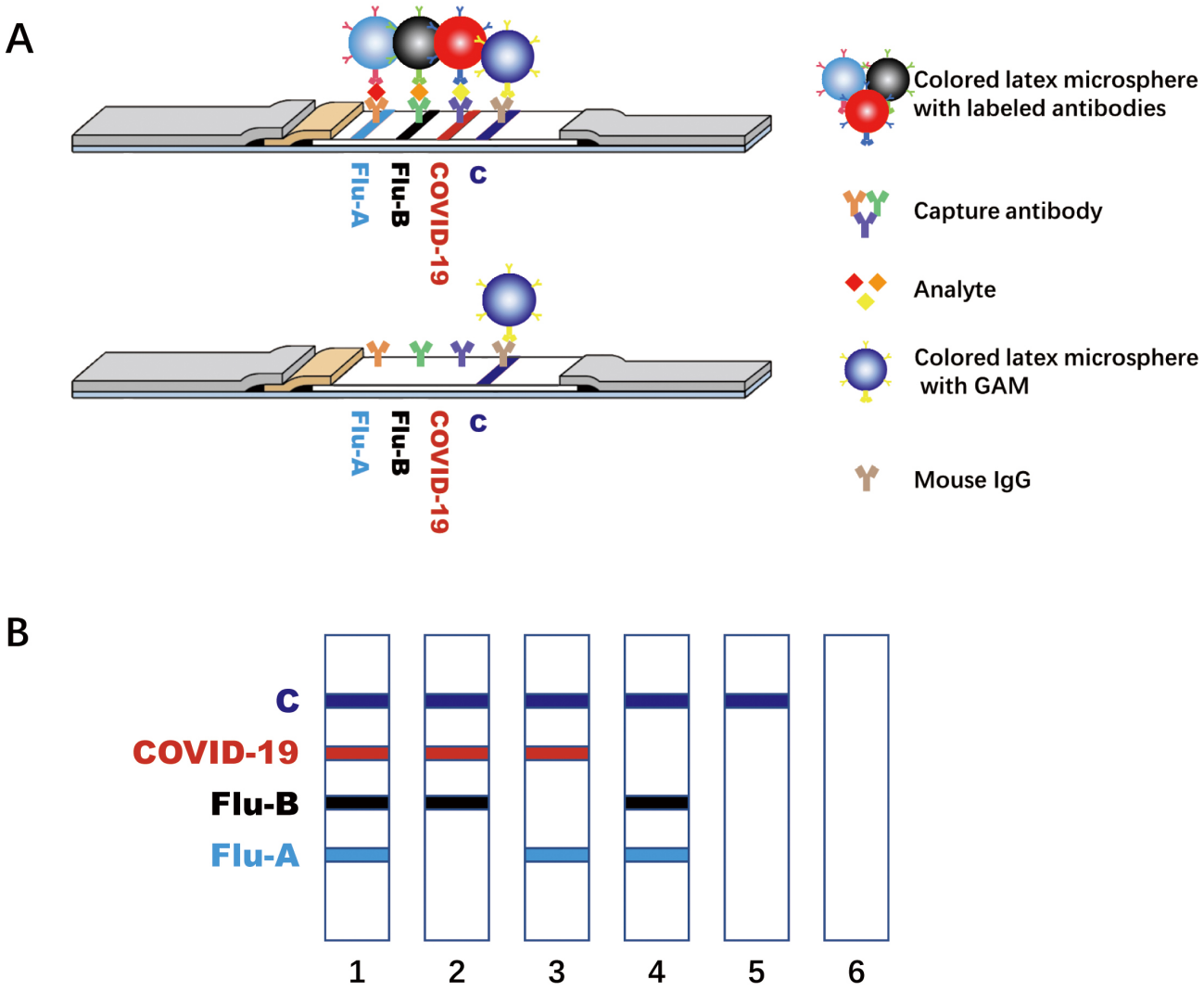

Fig. 1. The lateral flow strip structure: Schematic representation of the lateral flow strip. (A) Schematic illustration of a multicolor latex tracer-based lateral flow assay strip, in which three test lines of T1, T2 and T3 were designed for the simultaneous detection of SARS-CoV-2, influenza A and influenza B, and one C line confirms the validity of our method. (B) Schematic representation of the interpretation of qualitative test results. 1, SARS-CoV-2 (+), influenza A (+), influenza B (+); 2, SARS-CoV-2 (+), influenza A (-), influenza B (+); 3, SARS-CoV-2 (+), influenza A (+), influenza B (-); 4, SARS-CoV-2 (-), influenza A (+), influenza B (+); 5, SARS-CoV-2 (-), influenza A (-), influenza B (-); 6, invalid. "+" and "-" indicate the "positive" and "negative" results, respectively. The schematic illustrations were created using Adobe Illustrator CS6 (Adobe Inc., San Jose, CA, USA).

manufacturer's instructions. The paired nasal swab was assigned for SARS-CoV-2 PCR testing and influenza A and B antigen testing, which served as the comparator methods in this study. Swabs for PCR or antigen testing were collected and preserved by trained personnel according to the relevant instructions. For SARS-CoV-2 detection, clinical samples were analyzed using real-time reverse transcription polymerase chain reaction (RT-qPCR) following standard clinical diagnostic protocols. Viral RNA was extracted from respiratory specimens, and amplification was performed targeting specific regions of the SARS-CoV-2 genome (*ORF1ab* and *N* genes) [24]. A cycle threshold (Ct) value below the manufacturer-defined cutoff was considered positive. For IAV and IBV, antigen-antibody reactions were employed in accordance with established clinical diagnostic procedures [25,26]. The results of RT-qPCR were used as the reference standard for evaluating the performance of the developed immunochromatographic assay.

Statistical Analysis

All statistical analyses were performed using SPSS software (version 19.0, IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 6.01, GraphPad Software, San Diego, CA, USA). Sensitivity, specificity, and overall concordance rate were calculated using RT-qPCR results as the reference standard. The agreement between the

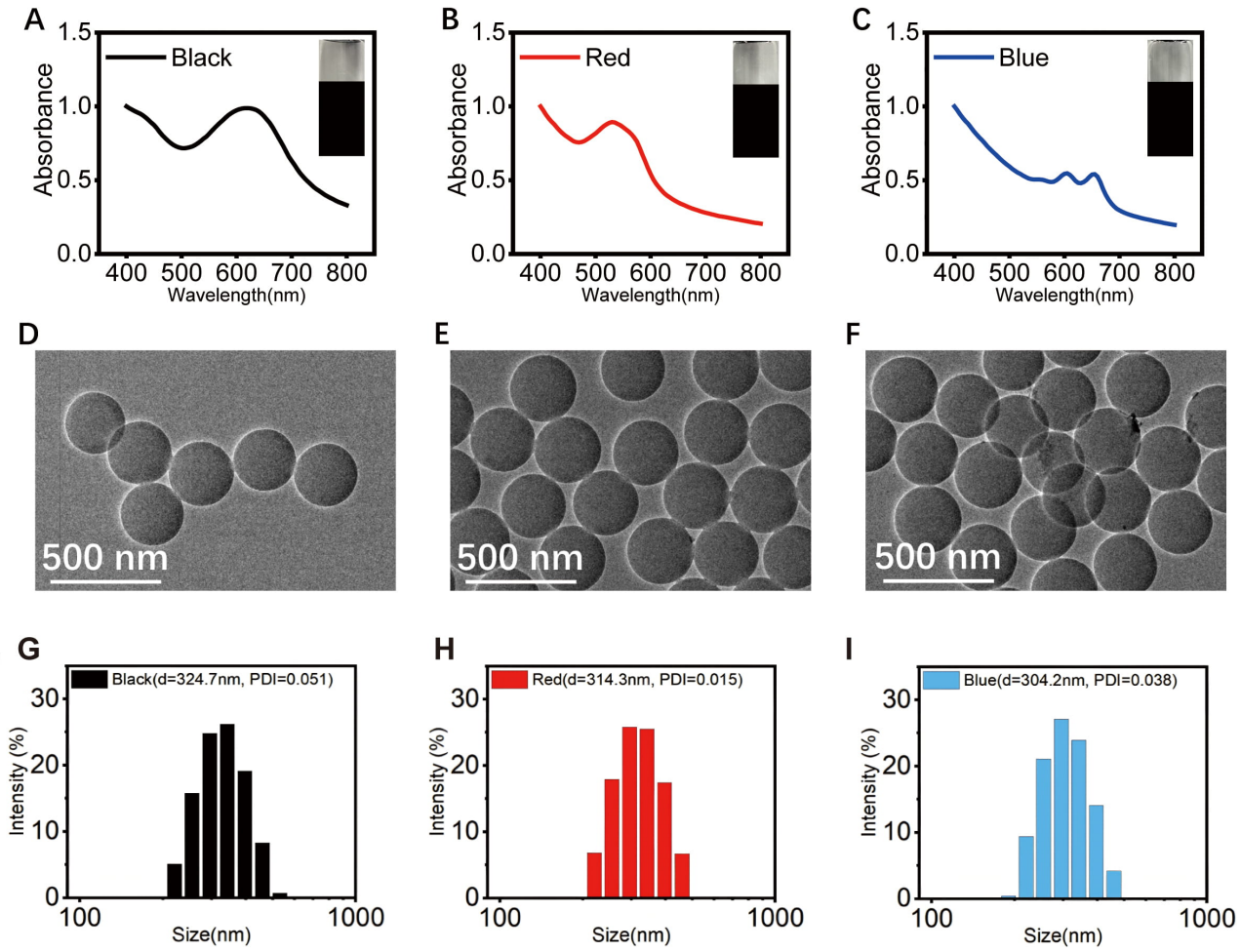

Fig. 2. Characterization of three differently colored latex microspheres. (A–C) Utraviolet visible (UV)-visible absorption spectra. Inset: the corresponding photographic images of the latex solution. (D–F) Transmission electron microscopy (TEM) images. (G–I) Hydrodynamic diameter. PDI indicates the polydispersity indices.

developed assay and the reference method was evaluated using Cohen's Kappa coefficient, with a value >0.75 indicating excellent agreement. For quantitative analysis, the test line to control line (T/C) ratios were calculated based on grayscale intensity measurements obtained using ImageJ software. Calibration curves were constructed by plotting T/C ratios against the logarithm of virus concentrations, and linear regression analysis was performed after logit transformation. Statistical significance was defined as $p < 0.05$.

Results

Detection Principle of the Multi-Color Latex Tracer Immunochromatographic Strip

The multi-color latex tracer immunochromatographic strip was developed based on the double-antibody sandwich assay. As shown in Fig. 1A, in the presence of the target analyte, the capture antibody on the strip surface captures the target analyte—detection antibody—multi-color latex microsphere complex and forms a sandwich structure, producing the signal.

In contrast, in the absence of the target analyte, the detection antibody–multi-color latex microsphere complex cannot be directly captured by the capture antibody, resulting in no detectable signal. With increasing target concentration, more multi-color latex microspheres were captured by the capture antibodies on the T lines, resulting in dark color bands on these lines. Thus, a directly proportional relationship between the color intensity at the T line and the analyte concentration in the sample could be observed. Regardless of the presence or absence of the target, the appearance of one C line signifies the availability of the multi-color latex tracer immunochromatographic strip.

Interpretation of results: Positive results: The presence of the Control line (C) and Test line (T) indicates a positive result. The red test line indicates a positive result of SARS-CoV-2 N protein antigen, the blue test line indicates a positive result of IAV antigen, and the black test line indicates a positive result of IBV antigen. Negative results: The presence of only the Control line (C) indicates a negative result. Invalid results: If the Control line (C) is not visi-

ble after performing the test, the result is considered invalid. Some causes of invalid results include failure to follow the directions correctly or deterioration of the test beyond its expiration date. It is recommended that a new test be used to retest the specimen. The interpretation of the results is shown in Fig. 1B.

Preparation and Characterization of the Color Latex

As shown in Fig. 2A–C, photographs of the colored latex microsphere suspensions under normal room lighting showed bright colors, and the UV-vis spectra of the colored latex microspheres showed different absorbance peaks. TEM images in Fig. 2D–F reveal a relatively uniform spherical nanostructure. Particle size analysis of latex microspheres and latex microsphere conjugates was conducted by dynamic light scattering (DLS) to characterize the particle size and light intensity distribution of latex microspheres [14]. As shown in Fig. 2G–I, the three colors of the latex showed comparable mean diameters. The latex microsphere solution system had good dispersion, indicating that the prepared latex microspheres had uniform particle size and a narrow size distribution.

Performance Evaluation of the Multi-Color Latex Tracer Immunochromatographic Strip

The multiplex detection ability of our newly developed multi-color latex tracer immunochromatographic strip was evaluated by analyzing different concentrations of the three viruses. The detailed detection results of each virus in the individual tests are presented in Fig. 3. As displayed in Fig. 3A–C, three differently colored T lines with one-to-one correspondence to the three viruses were clearly observed on the NC membrane. Specifically, the red line corresponds to SARS-CoV-2 detection, the blue line corresponds to IAV detection, and the black line corresponds to IBV detection. This one-to-one correspondence between T-line color and analyte presents immense potential for on-site and rapid response to line positioning and virus types when naked-eye-based visual analysis is conducted, effectively overcoming the limitations of traditional monochromatic multiplex immunochromatographic strips. Moreover, the color of the individual T lines gradually darkened with increasing virus concentration.

The LOD was determined by performing gradient dilutions of heat-inactivated virus cultures (**Supplementary Table 1**). SARS-CoV-2 was tested over a concentration range of 1.0×10^6 to 1.0×10^2 $TCID_{50}$/mL, while IAV and IBV were tested from 1.0×10^4 to 1.0×10^2 $TCID_{50}$/mL. All concentrations within these ranges yielded positive results, with consistent reproducibility across repeated measurements. Based on the predefined criterion (≥95% detection rate), the LOD of the assay was determined to be 1.0×10^2 $TCID_{50}$/mL for all three viruses. Within the concentration range evaluated in this study, neither signal attenuation nor abnormal band disappearance was observed, indicating that no apparent hook effect occurred under the tested experimental conditions (SARS-CoV-2, 1.0×10^6–1.0×10^2 $TCID_{50}$/mL; IAV and IBV, 1.0×10^4–1.0×10^2 $TCID_{50}$/mL).

The T/C ratios were used as quantitative signal outputs for accurate quantitative detection [27]. As illustrated in Fig. 3D–I, the standard calibration curves and linear regressions after logit transformation for each virus in the individual tests were created by plotting T/C ratio against target virus concentrations. Fig. 3 demonstrates that each T line specifically targets its corresponding virus, with no apparent cross-reactivity or inter-virus interference observed among the three viruses, even at high concentrations. The analytical specificity of the assay was further evaluated using five common non-target respiratory pathogens, including respiratory syncytial virus, Mycoplasma pneumoniae, parainfluenza virus, adenovirus, and rhinovirus. Detailed cross-reactivity results are summarized in **Supplementary Table 2**.

Clinical Evaluation

In this study, the samples were collected, and the results are shown in Table 1. The Kappa value of the consistency check for the SARS-CoV-2 test was 0.9762 ($p < 0.05$). The consistency of IAV and IBV antigen detection was 100%, indicating that the Three Antigen Test Kit had good agreement with the reference reagent. The test results showed that the novel coronavirus antigen detection kit developed in this study had high sensitivity, good specificity, a high coincidence rate, and good agreement with clinical diagnosis, and could be used as an effective screening tool for the diagnosis of SARS-CoV-2, IAV, and IBV infection.

Discussion

The rapidly spreading COVID-19 caused by SARS-CoV-2 is considered a public health emergency of international concern (PHEIC) [28]. With the arrival of the global influenza season, the international community may face a serious dual public health crisis, and respiratory pathogen screening in various countries and regions may face unprecedented challenges [29]. Therefore, it is important to develop specific and highly sensitive detection methods to quickly identify and manage COVID-19 and influenza patients and implement control measures to limit COVID-19 and influenza outbreaks.

While viral nucleic acid detection remains the “gold standard” for confirming infection [30,31], the influence of sampling time, instruments, reagent quality, laboratory quality management, and other factors makes molecular detection relatively expensive and time-consuming, making it unsuitable for large-scale screening programs [32]. IgM and IgG antibody detection can determine whether a patient has recently or previously been infected with SARS-CoV-2, which is helpful for the diagnosis of clinically sus-

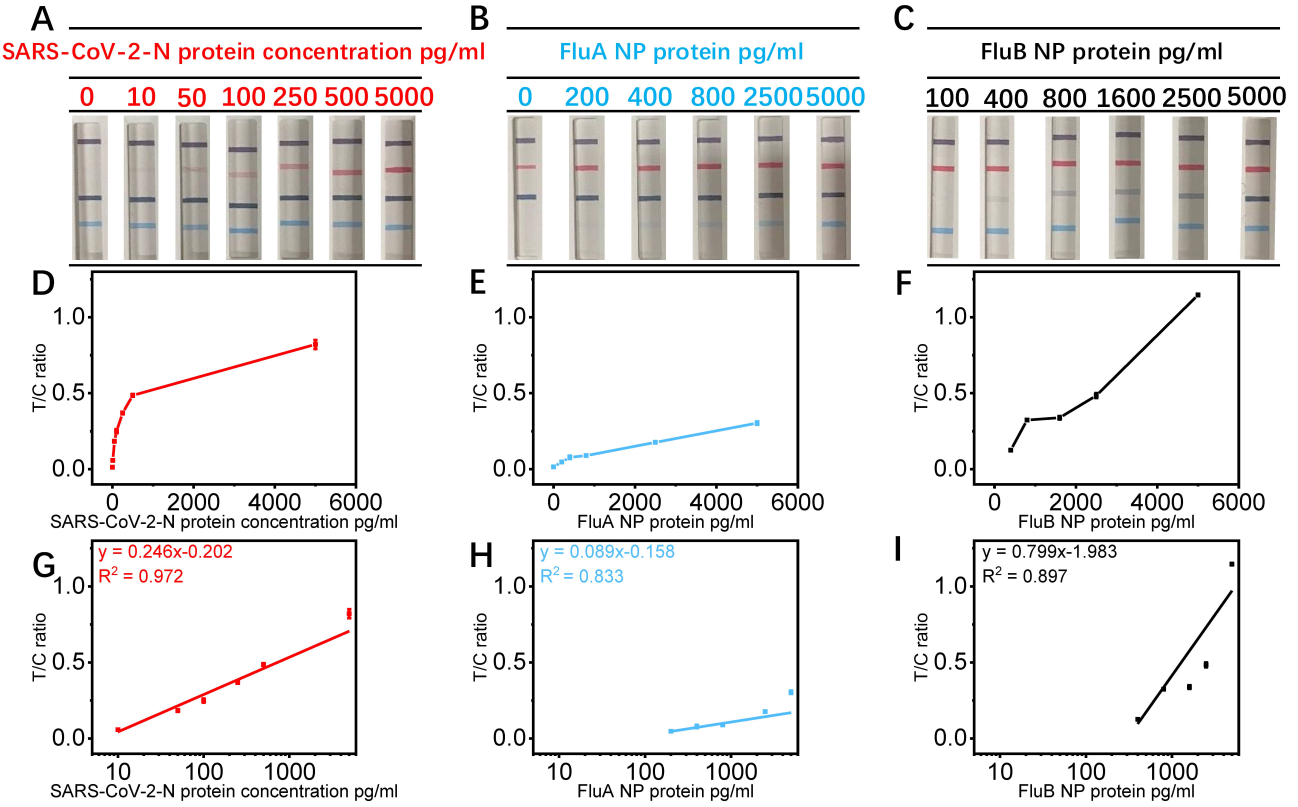

Fig. 3. The individual test of three viruses with our multi-color latex tracer-based immunochromatographic strip. Stereograms (A–C), calibration curves (D–F) and linear regressions (G–I) after logit transformation to individually detect SARS-CoV-2 (A,D,G), influenza A (B,E,H), and influenza B (C,F,I).

Table 1. Characteristics of clinical samples.

The three-antigen kit		Vitassay Healthcare S.L.U. kit (qRT-PCR)		Total
		Positive	Negative	
SARS-CoV-2	Positive	215	0	215
	Negative	5	200	205
Total		220	200	420

The three-antigen kit		BinaxNOW™ Influenza A&B Card kit (Immunochromatography)				Total
		Influenza A		Influenza B		
		Positive	Negative	Positive	Negative	
Influenza A	Positive	231	0	0	231	703
	Negative	0	472	272	200	
Influenza B	Positive	0	272	272	0	703
	Negative	231	200	0	431	
Total		703		703		1406

pected patients with negative nucleic acid detection. However, there is a window period for antibody detection. Specific antibodies generally appear 7 days after human infection, and antibody detection is vulnerable to interference, leading to false positives [33]. Antigen detection can compensate for the limitations of nucleic acid detection and antibody detection to a certain extent because of its convenience, rapid turnaround, and low cost. Antigen detection primarily relies on immunoanalytical methods. According to different markers, common methods include enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay (CLIA), and Lateral flow immunochromatography (LFIA) [34,35]. In this study, we captured three viral antigens using LFIA technology with three specific antibodies labeled with different colors of latex. The target viral antigen in the sample was recognized by a latex-labeled antibody to form an antigen-antibody complex, which then bound to an immobilized antibody

on the detection line to form an antibody-antigen-antibody complex (sandwich complex). Finally, the positive result was determined by the latex color signal, as illustrated in Fig. 1A. This sandwich format can overcome methodological limitations and improve the specificity and sensitivity of capture. In addition, highly specific monoclonal antibodies with sufficient affinity were selected to capture the SARS-CoV-2 N protein and the surface proteins of IAV and IBV, thereby improving detection performance.

Although RT-qPCR remains the gold standard for SARS-CoV-2 detection, with the continued maturation of SARS-CoV-2-specific antibody screening, many antigen detection products have shown improved detection performance, playing an important role in large-scale screening, close contact tracing, and emergency detection, and serving as effective adjuncts to RT-qPCR [36]. Especially in high-prevalence areas, the lower sensitivity of antigen detection compared with RT-qPCR can be overcome to some extent by adjusting the testing frequency appropriately [37]. Increasing modeling evidence suggests that the best way to control virus transmission is to expand the tested population, report results rapidly, and repeat testing [38,39]. Therefore, when RT-qPCR capacity is overloaded, antigen detection may be the best choice.

At present, COVID-19 infections and transmissions remain relatively high in the population, and effective diagnostic methods are still needed. With the approach of the influenza season and the recovery of influenza infection rates, detection strategies should be adjusted to enable simultaneous detection of influenza viruses and SARS-CoV-2. There is an urgent need to perform differential diagnoses of influenza and SARS-CoV-2 infections with similar symptoms, so as to improve the efficiency of diagnosis and treatment and support accurate medical management. This test kit provides a potential solution, as it can rapidly distinguish SARS-CoV-2, IAV, and IBV infections through color signals within 15 minutes, and can even identify patients with double/triple infections without the need for professional staff or laboratory facilities [40]. This product meets clinical and market needs and can be used in emergency rapid screening, community large-scale screening, entry-exit border inspection, and other scenarios. Compared with other similar kits, this kit showed a sensitivity of 97.73% and a specificity of 100% for SARS-CoV-2 antigen detection, demonstrating significant advantages. Evaluation of the kit showed favorable performance in terms of the limit of minimum detection, cross-reactivity, accelerated stability, specificity, and sensitivity, supporting its potential as an effective alternative to RT-qPCR.

This study has several limitations. First, the cross-reactivity panel did not include all possible respiratory pathogens. Although specificity against common and clinically relevant pathogens was demonstrated, future studies should include additional viruses (e.g., seasonal human coronaviruses and human metapneumovirus) to further validate assay specificity. Second, the assay performance was not directly evaluated using multiple SARS-CoV-2 variant strains. While the nucleocapsid (N) protein is relatively conserved and previous studies suggest that N protein-based antigen assays retain detection capability across major variants [41,42], the lack of experimental validation with variants may limit the comprehensiveness of the evaluation. Third, a separately defined healthy population cohort was not included for assessing false-positive rates. Although RT-qPCR-negative clinical samples were used as controls [10,43], inclusion of well-characterized healthy individuals would strengthen real-world specificity assessment. Fourth, viral samples were prepared using serial dilutions of inactivated virus cultures rather than clinical specimens with naturally distributed viral loads, and a fully validated quantitative model was not established. Although representative images demonstrated concentration-dependent signal variation, systematic quantitative analysis of signal intensity (e.g., T-line grayscale measurement or signal-concentration curves) was not performed, and the relationship between analyte concentration and signal response was not fully characterized. In addition, evaluation of the hook effect was limited to observations within the tested concentration range (10^6–10^2 $TCID_{50}$/mL for SARS-CoV-2 and 10^4–10^2 $TCID_{50}$/mL for IAV and IBV), where no evidence of signal attenuation or abnormal band disappearance was observed. A more comprehensive assessment would require detailed signal intensity profiling across an extended concentration range. Fifth, the effect of temperature on chromogenic reaction kinetics was not systematically evaluated. Although the assay was designed for qualitative detection under standard conditions [44,45], environmental factors may still influence signal development. In addition, the stability of the test strips under varying storage conditions was not assessed. Finally, user interpretation and usability were not formally evaluated. Although the multicolor labeling strategy improves visual discrimination by assigning distinct colors to each target analyte, no systematic user study was conducted to assess interpretation consistency among operators with different educational backgrounds. Therefore, potential variability in result interpretation under real-world conditions cannot be fully excluded. Future studies are warranted to address these limitations to further enhance the robustness and clinical utility of the assay.

Conclusion

In conclusion, the SARS-CoV-2, IAV, and IBV antigen detection kit developed based on three-color latex immunochromatography is a sensitive and accurate joint screening tool for respiratory pathogens. It provides rapid results, effectively distinguishes SARS-CoV-2 from influenza A/B infections, and supports timely identification of infected patients and close contacts, thereby helping control disease transmission.

Availability of Data and Materials

The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

LF, ZL, GL: conception, drafting the initial version, proofreading, interpretation of data, planning of lab experiments; LF, ZL: lab experiments; LF, GL: statistical analysis. All authors contributed significantly to editorial changes of important content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Ethical approval for this study was obtained from the Clinical Research Ethics Committee of the Clinical Laboratory of Tongxiang Zhouquan Town Central Health Center between 2024-2025 (Approval No. SBQLL-2025-475). As this study involved only the use of anonymized residual clinical samples and did not include any additional patient intervention, a waiver of informed consent was granted by the ethics committee in accordance with institutional policies and applicable regulations. All procedures involving human participants were conducted in accordance with the ethical standards of the institutional research committee and with the principles of the Declaration of Helsinki.

Acknowledgment

Not applicable.

Funding

This work was supported by the Scientific Research Project of Tongxiang City (Project No. 202402764).

Conflict of Interest

ZL was employed by Dian Diagnostics Group Co., Ltd. Other authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202638210.171.

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