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Editorial

As a result of the COVID-19 pandemic, the development of sensitive and accurate SARS-CoV-2 rapid point-of-care (POC) tests received much attention in society and in the greater medical and scientific communities. Rapid POC tests, such as lateral flow assays, are devices used to confirm the presence or absence of a target analyte, such as a pathogen (e.g., SARS-CoV-2) or biomarker (e.g., human chorionic gonadotropin - pregnancy test). These tests are designed to be inexpensive, easy to use with little to no equipment required, and yield diagnostic results at or near the point-of-care. Despite these advantages, one of the main challenges that has hindered the development of rapid POC tests has been the prevalence of false-positive and false-negative results. This issue underlies the importance of finding ways to enhance the detection sensitivity and specificity, to yield highly reliable, reproducible, and accurate rapid POC tests.

In the development of a lateral flow assay, there are several critical components that should be considered, which may affect the sensitivity and overall performance of the test. First, understanding the In Vitro Diagnostic Regulation (IVDR) and the regulatory approval process is important for the development and manufacturing of rapid POC devices. Knowledge of this information could influence decisions in the assay development workflow, underlying the importance of choosing the right product for the particular stage of development. Importantly, the manner of analyte and sample preparation can affect overall performance of the POC test. Maintaining the integrity of the sample is key to preventing the prevalence of false-positive or false-negative results. Also, the appropriate type of sample matrix (e.g., urine, saliva, mucus, serum) must be considered when choosing the optimal sample and absorbent pads. Another important component are the antibodies selected for the test; these reagents recognize and bind to the biological target of interest with affinity. Selecting the optimal type of antibody that is both sensitive and selective towards a low analyte concentration is a critical aspect of rapid POC test design. Finally, one must also consider the detection reagents chosen for the generation, visualization, and amplification of a signal from the analyte of interest. Some of the most common detection materials include colloidal gold nanoparticles, latex beads, enzyme conjugates, fluorescent particles, or magnetic particles. The chosen detection chemistry may be able to enhance the lateral flow assay and lower the limit of detection.

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Due to the importance of these elements, this booklet provides an overview of the development of rapid POC testing products. The studies discussed herein focus on the above-described aspects of rapid POC test development: IVDR/regulatory approvals, analyte and sample preparation, antibody selection, and detection chemistries. Following the article summaries, this booklet contains an interview with two leading experts in the field of rapid POC testing products: Matthew Coussens, Product Training Manager at MilliporeSigma, and Lisa Fitzpatrick, Diagnostics Field Marketing Manager at Merck KGaA, Darmstadt, Germany. These experts provide valuable insight and their own recommendations for designing and developing lateral flow assays. Through these article summaries and expert insights, we hope to educate researchers on important considerations and strategies to enhance the design and development of rapid POC tests.

Emily E. Frieben, Ph.D.,
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In vitro Diagnostics for Screening the Blood Supply: the New European Regulation for IVD and the WHO IVD Prequalification Programme

Mbunkah H.A., Reinhardt J, Kafere C, *et al*.

Article

n vitro devices (IVDs) are used extensively in determination of blood types for ensuring compatibility between donor and recipient and for screening for transfusion-transmissible infections (TTIs), i.e., to eliminate contaminated blood from blood donor pool. The World Health Organization (WHO) has noted that the safety level of donated blood globally can be lower when blood is not screened for all TTIs or the screening is not performed in a quality assured manner. Thus, specifications and guidelines for manufacturing and performance of IVDs involved in human diagnosis and assessment of medical treatments is warranted in all countries, including those with limited resources.

The new European Union (EU) Regulation for *in vitro* medical devices (IVDR, 2017/746/EU) replaced the previous *in vitro* medical devices Directive (IVDD, 98/46/EU) during a five-year transition period, ending May 26, 2022. The *In Vitro* Diagnostic Regulation (IVDR) comprises new rules and regulations that provide a transparent and sustainable framework for harmonizing the safety, quality, and performance of IVDs across the European Union. As of May 26, 2022, the new regulations need to be followed to remain compliant.

According to the regulations, the IVDR definition of an *in vitro* diagnostic device (IVD)

> '*In vitro* diagnostic medical device' means any medical device which is a reagent, reagent product, calibrator, control material, kit, instrument, apparatus, piece of equipment, software or system, whether used alone or in combination, intended by the manufacturer to be used *in vitro* for the examination of specimens, including blood and tissue donations, derived from the human body, solely or principally for the purpose of providing information on one or more of the following:
> - concerning a physiological or pathological process or state
> - concerning congenital physical or mental impairments
> - concerning the predisposition to a medical condition or a disease
> - to determine the safety and compatibility with potential recipients
> - to predict treatment response or reactions
> - to define or monitor therapeutic measures.

Specimen receptacles also are deemed to be *in vitro* diagnostic medical devices.

Examples of *in vitro* diagnostic devices include pregnancy tests and blood glucose monitors.

Briefly, the IVDR applies to *in vitro* medical devices for human use and its accessories, including laboratory-developed tests (LDTs). The IVDR also requires performance studies for the IVDs and its accessories. The IVDR describes independent certification organizations called "Notified bodies" that are set up by the countries of Europe. The Notified Bodies will be responsible for performing third-party assessment activities on the IVDs that include testing, calibration, inspection, and certification. Their findings will determine whether the IVD can be marketed in the member state and receives the "Communauté Européenne" (CE) mark. The key changes in the IVDR are listed in Figure 1.

In contrast, the IVDR does not apply to products for research use or general laboratory use, unless the products are specifically used for diagnostic examinations. IVDR does not apply to internationally certified reference material, nor to invasive sampling products or products used to obtain specimens from a human body. IDVR also does not apply to materials used for external quality assessments.

The IVDR used risk of failure for the individual patient and for public health to categorize the LDTs into four classes (Table 1). Group A LDTs had low risk of harming individuals or disrupting public health whereas Group D LDTs (eg, diagnostic

IVD tests for HIV or Hepatitis B Virus (HBV)) that fail pose a high risk of harm to not only individuals but also to public health. Examples for each risk group are listed in Table 1. Manufacturers of IVDs in risk groups B, C, and D need to send information to the Notified Body for their approval before obtaining a CE mark.

Prequalification of IVDs by World Health Organization

The WHO, while not a regulatory body, helps member states to select essential health products including IVDs by providing prequalification assessments for their decision-making process. Mainly, the IVDs for the diagnosis of life-threatening diseases such as HBV, HCV, HIV and malaria can be prequalified for a limited resource environment by the WHO.

- Eligibility for prequalification of IVD by WHO reflects a combination of the following factors:
- Global need for IVDs for particular disease state or disease
- Product appropriate for use in resource-limited environments
- WHO recommendation in disease-specific guidelines
- Requests by member states
- Available prequalified products with similar assay principle and/or format

Figure 1

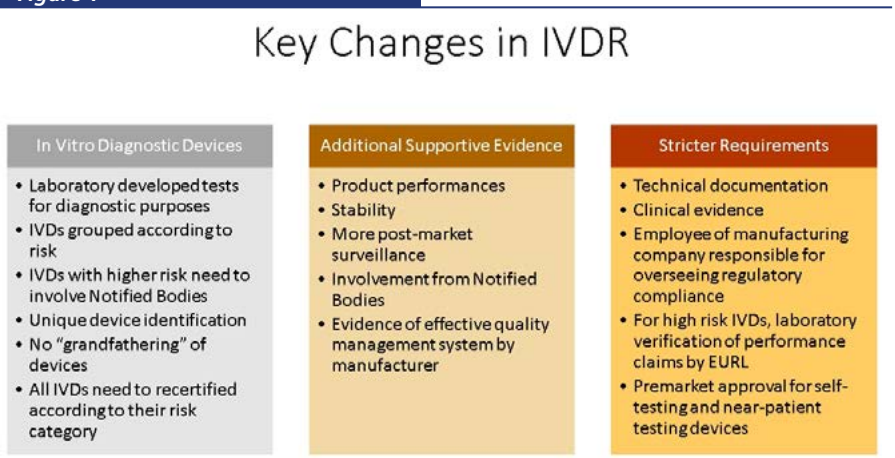


Fig. 1: *Key changes in European IVDR compared to the previous IVDD regulation. EURL, European Union Reference Laboratory; IVDR, in vitro device regulation in European Union; IVDs, in vitro devices.*

WHO welcomes applications for prequalification from manufacturers of IVDs for the very high-risk TTIs such as HIV, HBV, HCV, but also for IVDs for other pathogens such as Vibrio cholerae, human papilloma virus (HPV), malarial parasites, and G6PD enzyme activity.

The manufacturer begins the IVD prequalification (IVD-PQ) process with the WHO with a pre-submission form, which briefly provides information about the product, details of the manufacturer, and the product's regulatory version. If the IVD-PQ team deems the product is eligible for assessment, then the manufacturer compiles a product dossier which covers at least the following issues:

- Product's description including its principles, and efficacy with variants
- Risk analysis and control
- Manufacturing information
- Product performance specifications
- Validation and verification studies of product performance
- Labeling
- History of commercial availability and usage

Table 1

Group	Risk of failure	Notified body?	Examples (Includes reagents, reagent products, control materials, related calibrators, software)
A	Low individual risk Minimal public health risk	No	Receptacles for specimens Instruments for IVD procedures
B	Moderate individual risk Low risk to public health	Yes	Near patient testing in health institutions Self-testing for cholesterol level, fertility, pregnancy, In urine, self-testing for glucose, erythrocytes, leucocytes, and bacteria
C	High risk to individual Moderate risk to public health	Yes	Testing for following blood groups: anti-Duffy and anti-Kidd Testing for irregular and anti-erythrocytic antibodies Testing for congenital infections of toxoplasmosis, rubella Testing for phenylketonuria (hereditary) Testing for HLA tissue groups, DR, A, B Testing for human infections chlamydia, cytomegalovirus Testing for Prostate specific antigen (PSA) Testing for measurement of glucose Testing for risk of trisomy
D	Life-threatening risk to individual; Widespread risk to public health	Yes	Testing for following blood groups: ABO system, Rhesus (C, c, D, E, e), anti-Kell IVDs for detection, confirmation and quantitation of high-risk infectious diseases in human specimens: HIV 1 and 2, Hepatitis B Virus (HBV), HCV, HDV, HTLV I, HTLV II
HTLV I, human T-lymphotropic virus type I; HTLV II, human T-lymphotropic virus type II.			

Table 1: *The IVDR categorized the current IVDs based on risk of failure in individuals and risk to public health*
**This list is not exhaustive to the examples column in table 1*

- Regulatory history
- Description of quality management system

The IVD-PQ team concurrently reviews the dossier and performs a manufacturing site review which assesses compliance of the quality management system and manufacturing practices with the ISO 13485: 2016 international standards. Briefly, the key personnel at the manufacturing site who run the quality management system, production line, and quality control must be present on the date of inspection. The site must be manufacturing at least one of the products being reviewed. Inspectors are usually invited from the local national regulatory authority to act as observers. The information in the dossier should agree with the observations at the manufacturing site.

In addition, the WHO performance evaluation laboratory assesses the performance characteristics and the operation of the product. The WHO reference panel includes well-characterized seroconversion panels, low titer panels, and geographically diverse plasma/serum specimens. Different laboratories evaluate the products for different infectious agents: for example, the WHO Collaborating Center in London focuses on evaluations of test kits for Hepatitis B virus and HCV. As expected, post-market surveillance obligations ensure that the prequalified manufactured IVD products continue meeting the same safety, quality, and performance requirements as during their prequalification assessments. The WHO evaluation also focuses on the transportation, and storage parameters for the IVDs because conditions may be suboptimal for the stability of the IVDs in resource-limited environments.

Regulatory cooperation occurs within some regions of the world. For example, one or more countries with limited resources may opt to accept certificates issued by regulatory body from country B, without repeating the full audit process: this process has been called recognition.

These IVD regulations in Europe and WHO encompass the product's whole life cycle in an effort to ensure a safe blood supply.

Competing Policy Windows in Biotechnology: The FDA, the 21st Century Cures Act, and Laboratory-Developed Tests

Myers N., Steding C.E., Mikolaj P.

Article

The authors researched the processes for developing US Food and Drug Administration (FDA) policy as related to laboratory-developed tests (LDTs). They reviewed comments and transcripts and identified the following three main groups who had provided comments, suggestions for policy initiatives, and insights on regulation of LDTs:

- People with a promotional orientation toward biotechnology
- People with a more protective focus toward biotechnology
- Certain members of Congress

They also identified themes that suggest tension between the objectives of the FDA guidance document called "Framework for Regulatory Oversight of Laboratory-Developed Tests (LDTs)" and the 21st Century Cures Act. They provide this case study that examines the evidence for competing windows of opportunity and its potential effect on the developmental process.

They observed that regulators claim oversight is necessary to maintain safety, whereas elected officials often view regulation as an obstacle, which should be removed or minimized. Many Congress members have shifted their mission on biotechnology from a protective regulatory emphasis to promoting biotechnology for generating new medical treatments and economic development.

The 21st Century Act

The objective was to ensure that regulations and laws kept pace with scientific progress. The Act contains provisions to:

- Address unmet medical needs, (eg, faster development and approval of antibiotics)
- Strengthen FDA to help develop and deliver new products
- Improved delivery of innovations for patient health such as
  - encouraging continuing education for medical professions,
  - improving interoperability of electronic health systems, and
  - making reforms to Medicare and Medicaid.

Although debated and discussed, no legislation on regulating LDTs was included in the 21st

Table 1

Organization	Comment
Advanced Medical Devices	• We commend FDA's efforts to take this critical step to support patient care and robust product development while ensuring that well-recognized gaps in oversight are addressed. . . • Patient-centered, risk-based regulation that facilitates innovation for safe and effective diagnostics is of paramount importance. • A test is a test and presents the same risk for patients regardless of whether it is developed by a traditional manufacturer or a laboratory. Potential harms to patients whose tests return incorrect results include unnecessary treatments with their accompanying costs and side effects and treatment delay or failure to obtain appropriate treatment, all of which lead to worse outcomes for those patients.
AHA – American Stroke Association	• We support the enhanced oversight proposed in the draft guidance documents and believe it is fundamental to ensuring that new discoveries are translated into reliable informational tools for health care professionals that can ultimately improve health outcomes for patients.
ASRO- American Society for Radiation Oncology	• We support the enhanced oversight proposed in the draft guidance documents and believe it is fundamental to ensuring that new discoveries are translated into reliable informational tools for health care professionals that can ultimately improve health outcomes for patients.
Friends for Cancer Research	• As organizations representing the interests of patients to whom access to high quality molecular diagnostics is a top priority, we would like to voice our support of the principles regarding oversight of tests used to inform diagnosis and treatment decisions described in the US Food and Drug Administration's recently published draft guidance entitled "Framework for Regulatory Oversight of Laboratory-Developed Tests (LDTs). . ."

Table 1: *Examples of comments from 4 of the 11 organizations who support FDA Guidance Document*

Table 2

Organization	Comment
AACC: American Association for Clinical Chemistry	• AACC strongly recommends that the FDA gather the data first before making decisions regarding the framework for LDT oversight. • AACC does not believe the adverse event framework, which was developed for reporting problems involving medical devices, is appropriate for services provided by clinical laboratories. • Results from LDTs do not generally result or contribute to the death or serious injury to a patient.
ACLA: American Clinical Laboratory Association	• On behalf of the undersigned organizations, which represent a broad and diverse array of stakeholders including, but not limited to, hospitals, clinical laboratories, physicians, other health care providers and industry involved in delivering medical care to millions of patients daily, we are writing to request that the Food and Drug Administration (FDA) withdraw the proposed draft guidance, "Framework for Regulatory Oversight of Laboratory-Developed Tests" and associated guidance.
Duke Center for Innovation Policy	• Administrative efficiency, and concern for undue negative impact on private sector innovation, also suggest that the guidance could be narrower in scope. • In fact, conventional administrative law principles strongly indicate that an FDA decision of this nature and magnitude should be implemented through notice-and-comment rule making. • In general, small, entrepreneurial enterprises may be disproportionately harmed by mandatory FDA review. Recent changes in the intellectual property regime combine with the prospect of FDA review to further diminish prospects for small players. • A requirement for FDA review may also be problematic for competitors of tests that are already approved by the FDA. The act of seeking FDA review could alert holders of patents associated with approved tests to the possibility of patent infringement, chilling competitor investment even when the underlying patents are not necessarily valid.
Stanford University Genomics Research	• These comments focus solely on possible, strongly negative consequences for genomics research of the Framework laid out in the Draft Guidance. They do not address issues raised by the Framework for clinical use of LDTs. We also focus on issues affecting human genomics research, though it is clear to us that the Framework could, depending on its interpretation, hold back many promising kinds of research in molecular biology that do not focus on human genome sequences, such as work focusing on RNA transcription, on methylation, on telomere length, on microbiomes, and so on. These comments should be read in light of that broader context. • The Draft Guidance has at least the potential to obstruct much safe and potentially invaluable research in human genomics, as well as other related fields in human molecular biology, by requiring researchers to obtain IDEs in situations where the IDE serves no purpose. Three different relatively minor interpretative or substantive changes in the Draft Guidance could eliminate those problems. We strongly encourage FDA to make at least one of our recommended changes, and preferably all three.

Table 2: *Segments from Comments from 4 of 23 organizations that called for Withdrawal or Major Revision of the New FDA Guidance Document*

Century Act. This article used multiple streams theory of policy making to look for any competing windows of opportunity that stalled action on regulation of LDTs. They also considered whether including multidisciplinary perspectives would help resolve the competing issues.

Regulation, Wicked Problems, Policy Windows, and Interdisciplinary Solutions

The regulation of biotechnology is complex as the FDA is not always viewed as a protective force. LDTs are emerging technologies and their usage varies widely: usage of results from LDTs can range from research only at a particular facility to confirming variants suggestive of diagnosis of genetic disease(s) not only at the parent facility but in some cases worldwide. The authors suggest that developing workable regulations for LDTs act as "wicked problems" in policy making.

Wicked problems are defined as having unique characteristics that are difficult to delineate and may reflect underlying societal discrepancies or larger societal challenges. Policy makers often have limited options to address them. Policy solutions for a wicked problem are often considered one-shot propositions with no way to test the solution before implementation. Congress is trying to support LDTs as a means toward increasing economic development and generating new medical treatments while also protecting the public. The authors consider the development of balanced regulation for LDTs a wicked problem.

Interdisciplinary Collaboration

Several groups have argued for more engagement among scientists and policy makers that fosters a balanced transparent communication strategy. This approach may support increased protection of research participants and may help increase the acceptability of research into population genetics. In contrast to "experts and scientists" providing information to the general public which the public may not believe, a bidirectional communication approach should be used among citizens, decision makers, and experts to help create mutual understanding of personalized medicine and genetic testing.

Note that experts with different perspectives must be allowed to voice their insights and concerns. Otherwise, the general public may interpret the "chosen expert opinion" as an agenda. Science is built on the testing of ideas and continued discussion of the interpretation of observations.

The authors suggested that interdisciplinary communication strategies may be very useful in discussing the potentially competing interests (promotion of biotechnology and protection of public) while shaping potential regulation of LDTs, including

consumer genetic testing. Persuasion in writing and speaking are keys to success in the pollical arena.

Kingdon's theory of policy streams helps stakeholders, scientists and citizens examine the competing policy phenomena. The authors suggest that advocates for biotechnology innovation and increased funding helped stimulate the writing of the 21st Century Cures Act by persuading the policy makers of

1. Already thriving biotechnology industry in the US
2. Aging population with likely genetically linked diseases in need of identification of cause and new treatments

In an analogous manner, advocates for research participant and patient safety argued for increased regulation (FDA guidance) while acknowledging the growth of the biotechnology industry and need for innovations.

Methods

This study uses the transcript from the House Energy and Commerce Committee hearing, media accounts, public comments submitted to the FDA, academic literature, and reports from nonprofit organizations to investigate the idea of competing policy windows during development of regulation of LDTs. Supportive comments in favor of regulatory guidance were obtained from 33 organizations and individuals which included their company letterhead. Examples of supportive comments of the new FDA guidance document are listed in Table 1.

Twenty-three organizations wrote comments calling for revision or withdrawal of the FDA Guidance document (Table 2). Seven organizations called for clarification, more information, or minor revisions of the FDA Guidance document (Table 3).

Questions of Authority and Resources

The hearing brought up multiple issues in the development of more active FDA regulation of LDTs. The first theme was the "statutory authority to regulate LDTs." Dr Shuren of the FDA argued that the FDA has had the authority and had previously chosen not to exercise it. Both the AHA and the Advanced Medical Devises organizations supported the FDA's position of authority.

In contrast, the American Clinical Laboratory Association argued that LDTs are not manufactured products but are part of a clinical process, in agreement with comments from the American Society for Clinical Pathology, the AMA, and the University of Florida School of Medicine.

The second theme questioned whether the resources at the FDA would be sufficient to administer the additional regulations. Historically, the FDA approved less than 25 premarket applications for diagnostic tests whereas over 100,000 LDTs may come under its jurisdiction. The College of American Pathologists recommended that low and moderate risk LDTs be evaluated by third-party inspectors so FDA could focus on high-risk LDTs.

The third theme raised overarching questions about whether government regulation acts as a protection for patients or whether it's an unnecessary obstacle on innovation in the private sector, especially in the rare genetic disease field.

Several groups recommended cultivating objective policy analysts to help mediate the negotiated rule making, joint fact finding, and other collaborative policy-making processes.

Table 3

Organizations	Comments
Janssen Research and Development	• Frequent, transparent communications and focused, ongoing stakeholder engagement will be critical to the success of Framework's implementation. Whereas the Draft Guidances have capably illuminated FDA's general intentions for LDT oversight and the proposed parameters within which this oversight will occur, LDT developers will require greater clarity on many points over time.
Juvenile Diabetes Research Foundation	• It is important that research involving assays in the context of a drug or biologic therapy not be affected by this draft guidance. While we understand that assays used in the context of interventional studies of drugs and biologics, but not the subject of the study, are different from LDTs, there are similarities. In the current draft, it is unclear how this guidance relates to such assays. The assay may have been developed in one laboratory or within an LDT paradigm and therefore it is important for the developer to clearly understand the regulatory requirements as they develop the body of evidence needed for that assay to be used within the context of a drug or biologic clinical trial. We suggest that language be added to clarify that this guidance does not apply to assays that may be used in the context of drug or biologic studies. • For the categories included in the framework, the agency should clarify the requirements if a device falls into more than one category. • As laid out in Section D of the draft guidance and summarized on page 30, FDA has proposed various categories of LDTs with different levels of requirements FDA intends to enforce. The draft guidance does not address the requirements if a device falls into more than one category. For example, an LDT that tests a person's risk for developing T1D would be considered an unmet need, but could also fall into a category that FDA states is of higher concern to them. We anticipate there may be other cases of devices falling into more than one category. We suggest the agency include in the final guidance what the procedure will be when this happens.
PhARMA	• In conclusion, PhRMA remains supportive of FDA's proposal for a risk-based approach for LDT oversight. Due to the substantive changes being proposed and the clarifications requested, PhRMA urges the FDA to release a second version of the draft guidance.

Table 3 continued

Organizations	Comments
QIAGEN	• Although the draft guidance document provides clinical laboratories that manufacture LDTs a consistent description as to how FDA intends to enforce regulations that apply to such laboratories as medical device manufacturers, we have identified a number of points where additional clarity is necessary, especially as it relates to the obligations of traditional *in vitro* diagnostic (IVD) test manufacturers. We encourage FDA to apply the same submission, review and enforcement policies to all IVD manufacturers, regardless of type.
Personalized Medicine Coalition (PMC)	• Along with many others, PMC has requested additional information on risk classification, harmonization between the CLIA program and FDA QSRs, how technical test modifications would be handled, and labeling issues. • Alone, each of these issues is significant; yet together it is clear that, at the very least, a second draft of the Framework should be issued together with draft guidance documents clarifying the missing pieces for the review and public engagement process to be complete.. . . • Specifically, we request that FDA resolve outstanding issues, publish draft guidance documents on risk and CLIA–FDA harmonization, open a docket for the collection of public feedback and engage in a series of public engagement activities such as a webinar and public meeting.
AABB: American Association of Blood Banks	• For example, in transfusion medicine the search for compatible blood may result in a laboratory creating an antibody identification panel consisting of licensed reagents from more than one manufacturer. Is this an LDT? If so, how can the lab provide a timely notification? At the completion of the test, if the technologist decides to run the panel again using different incubation times, temperatures, or incubation or wash reagents, does this constitute a significant change to an LDT such that an additional notification is required? Again, how can the lab provide a timely notification? • The recommendation to report adverse events associated with a laboratory test or procedure through the MDR system is overly burdensome and without additional benefit to patients, donors, clinicians, or government agencies viewed in the context of already highly regulated blood establishments. • We believe that the current level of adverse event reporting that blood establishments are required to comply with is sufficient and recommend that Medical Device Reporting not be added.

Table 3: *Segments of comments from 6 of the 7 organizations that called for more information, clarification or minor revisions in the FDA Guidance Document*

Recent Applications of Paper-based Point-of-Care Devices for Biomarker Detection

Suntornsuk W and Suntornsuk, L

Article

Brief history of paper-based analytical devices (PADs)

Paper as a substrate or platform is used in development of many point-of-care (POC) tests, including lateral flow assays (LFAs). PADs offer many benefits including cost-effectiveness, portability, ubiquity, biodegradability, efficient wicking properties, low solvent consumption, and versatility in fabrication. The most commonly used paper in 2020 is Whatman filter paper grade 1. Other types of paper include Millipore MCE membrane filter and nitrocellulose.

A paper device usually contains at least these four regions: an inlet for sample loading, a microchannel that guides liquid flow, a barrier to maintain liquid within microchannel, and a location to support and read the chemical or biochemical reaction. Fabrication of a PAD in a hydrophilic paper matrix usually includes the construction of a hydrophobic barrier that directs liquid flow of reagents and analytes in a microchannel and prevents leaking or mixing of liquid. Both chemical modifications and physical deposition have been used to create a barrier.

Fabrication of 2D and 3D-PADs have used wax printing, flexographic printing, inkjet printing, screening printing, wax-screen printing, photolithography, laser treatment, plasma treatment, wet etching, and recently lacquer spraying, direct spraying with parafilm embedding, pen-plotting deposition method, and ink stamping.

Detection techniques used in PADs include electrochemical, colorimetric, fluorescent, photochemical reactions, and chemiluminescence. Optical signals such as chemiluminescence, fluorescence, absorbance, and transmittance are usually captured by specialized instruments (eg, camera, laser, microscope, scanner, spectrophotometer). Because these methods often use laboratory equipment not usually present in POC facilities and may require highly skilled operators, detection methods that do not require specialized equipment are often pursued for POC tests.

Colorimetric signals are commonly used in PADs and can be read by the naked eye. However, disadvantages may include lower sensitivity and specificity due to heterogeneous color distribution and inadequate lighting.

A distance-based detection PAD involves positivity (colorimetric changes) in the length of a bar which is inversely proportional to the concentration of the analyte [https://doi.org/10.1016/j.snb.2017.12.197]. Distance-based detection can improve consistency in reading of output. A scale ruler is provided and the signal can be read by the naked eye (Figure 1). Carcinoembryonic antigen (CEA) levels in serum were detected as low as 5 ng/mL with limit of detection being 2 ng/mL. Advantages of this semi-quantitative method include ease of operation, low cost, disposability, and general applicability for POC tests.

Analytes

The common sample types containing analytes include blood and blood derivatives (eg, serum, plasma, whole blood), urine, saliva and artificial saliva, and dilution in standard solution. Protein analytes can range in size to 200 KDalton (eg, carcinoembryonic antigen (CEA)) or larger. The platforms of the PADs need to take into consideration the viscosity of the sample, sample processing, and the pore size in the chosen paper to ensure sufficient flow of the analyte-capture antibody complex through the device in a timely manner.

PADs are commercially available to measure many types of biomarkers, including albumin, creatinine, cardiovascular disease biomarkers (eg, glycogen phosphorylase isoenzyme BB (GPBB), creatinine kinase MB (CK-MB), cardiotroponin T (cTnT)), glucose, lipids (triglyceride, high (HDL) and low-density lipoprotein (LDL)), and tumor biomarkers (eg, CEA, prostate specific antigen (PSA)) (Table 1).

Multiple analytes which have no cross reactivity can be measured by using a microfluidic paper-based device formatted for multiplex detection (Figure 2). As an example, Lim and coworkers [https://doi.org:10.1016/j.bios.2018.12.049] developed a PAD for diagnosis and prognosis of acute myocardial infarction. The PAD measured three biomarkers for cardiac injury (creatinine kinase isoenzyme MB (CK-MB), cardiotroponin T (cTnT), and glycogen phosphorylase isozyme BB (GPBB)). The PAD was fabricated on nitrocellulose paper by using wax printing to provide three microchannels in a branched flow pattern (Figure 2). The specific detection antibodies for CK-MB were conjugated to gold urchin AuNPs, those for cTnT to AuNPs, and those for GPBB to AgNPs to provide visible purple, red, and gold color spots respectively. Signals were captured by a phone

Figure 1

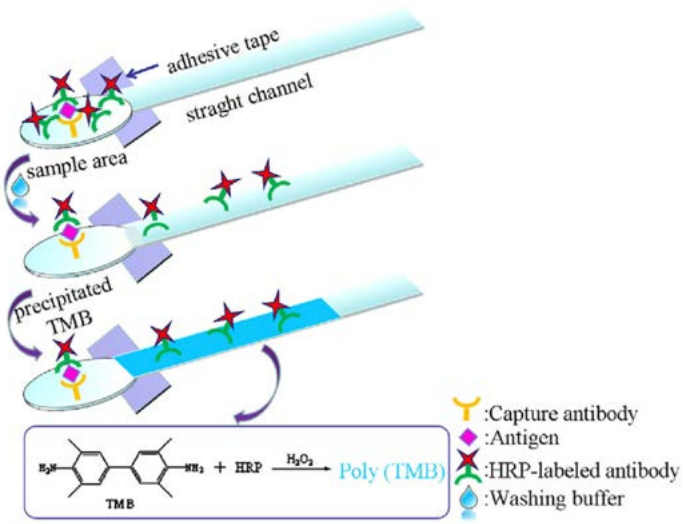


Fig. 1: *Schematic illustration of PAD that uses distance-based detection. Procedure included sequential addition to the sample pad of the capture antibody, sample containing the analyte, and detection antibody (horseradish peroxidase-labeled detection antibody. Additional steps included the washing buffer that propelled nonspecific antibody to flow into the distance detection channel and interaction of the TMB-H2O2 substrate with the HRP-conjugated detection antibody that provided a blue polymer in the straight channel.*

camera. Limits of detection for CK-MB, cTnT, and GPBB were 0.5 ng/mL, 0.05 ng/mL, and 0.5 ng/mL, respectively. The assay showed good correlation with the standard Siemens Centaur XPT Immunoassay system (R2=0.96). The short detection time (10 min) allows rapid measurements of these three risk factors in a POC device.

Modifications of substrate paper can improve binding efficiency of biomolecules.

Asthana and coworkers investigated different modifications of the paper format for their effect on sensitivity and specificity of the PADs [https://doi.org/10.1016/j.snb.2019.01.064]. The PADs used Whatman filter paper grade 4 printed with a wax printer. To potentially improve the characteristics of paper platform, the surface of different PADs was modified with AuNPs, chitosan, 3-aminopropyltriethoxysilane (APTES) and AuNPs with APTES (Figure 3). The paper modified with APTES and AuNPs exhibited a smoother surface than

Table 1

Analyte	Matrix	Material/ Structure	Fabrication technique	Detection technique	Range	Reference
Albumin	Whole blood	Filter paper/3D	Wax printing	CM	3.68-81 mg/mL	https://doi.org/10.1016/j.snb.2018.07.010
Bilirubin	Serum	Filter paper with ISM/2D	Wax printing	PD	5-500 μM	https://doi.org/10.1016/j.bios.2018.10.055
CA-125	Serum	Cellulose paper/2D	Screen printing	EC-IA	0.1–200 U/mL	https://doi.org: 10.1016/j.bios.2019.03.063
CA-15.3	Plasma	Photographic paper/2D	Inkjet printing (Ag/rGO)	ChA	15-125 U/mL	https://doi.org/10.1016/j.microc.2019.01.018
CEA, NSE	Serum	Chromatographic paper/2D	Wax and screen printing	IA-DD	0.01-500ng/mL 0.05-500 ng/mL	https://doi.org:10.1016/j.bios.2019.04.032
Creatinine	Urine	Filter paper/2D	Laser-jet printing	CM	0.025-0.25	https://doi.org/10.1016/j.talanta.2015.07.040
Dopamine	Blood, urine	Chromatographic paper/2D	Wax-based stamping	CV	0.5-120μM	https://doi.org/10.1016/j.sbsr.2019.100270
Glucose, Potassium iodate	Saliva	Filter paper/2D with was vales	Wax printer	Distance-based CM	1-5mg/dL 0.05-0.5 mM	https://doi.org:10.1021/acs.analchem.8b05764
GPBB, CK-MB, cardiotroponin T (cTnT)	Serum	Nitrocellulose membrane stacked with chromatographic paper/2D	Wax printing	CM	0–200 ng/mL 0–100 ng/mL 0–100 ng/mL	https://doi.org:10.1016/j.bios.2018.12.049

Ag/rGO, silver nanoparticle-reduced graphene oxide nanocomposite; CA-125, cancer antigen-125; CA-15.3, carbohydrate antigen 15.3; cTnT, cardiotroponin T); ChA, chronoamperometry detection; CM, colorimetric detection; CK-MB, creatinine kinase isoenzyme MB; CV, cyclic voltammetry; GPBB, glycogen phosphorylase isoenzyme BB; IA-DD, immunoassay distance-based detection; PD, potentiometric detection.

Table 1: *Characteristics of PADs for measuring a variety of analytes*

Figure 2

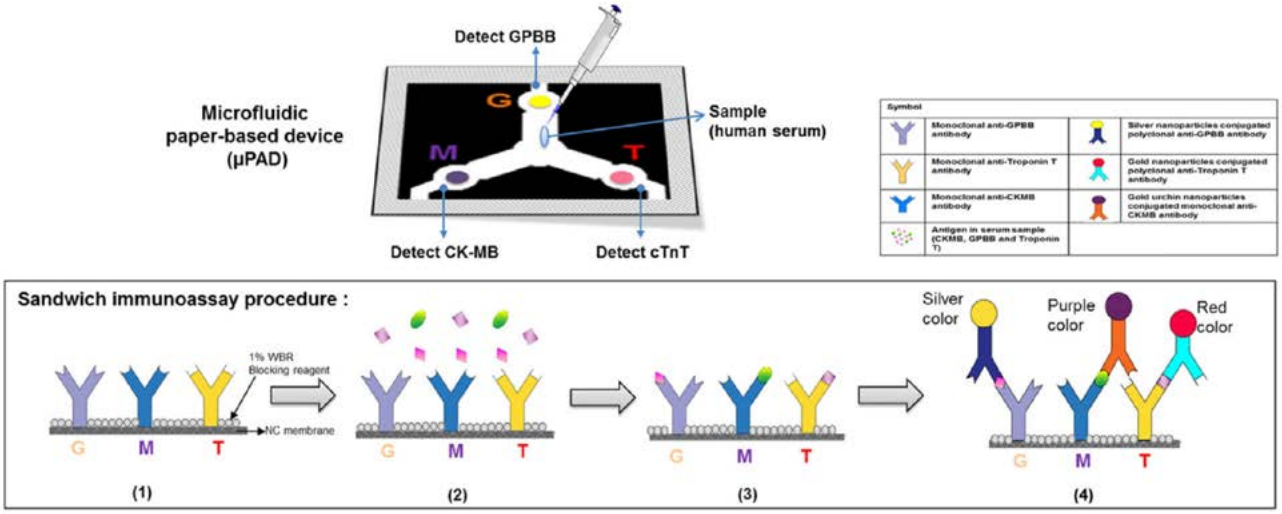


Fig. 2: *Fabrication scheme of multiplex μPAD for detection of the three cardiac markers: creatinine kinase isoenzyme MB (CK-MB), cardiotroponin T (cTnT), and glycogen phosphorylase isozyme BB (GPBB). A sandwich immunoassay procedure included (1) the immobilization of capture antibodies for the three risk factors at indicated sites (M, T, and G), (2) addition of sample to central zone, (3) capture antibodies bound target analytes in their respective zones, (4) detecting antibody for each analyte bound to target analyte-capture antibody complex, (5) color signals generated by the nanoparticle-conjugated detecting antibodies: The specific detection antibodies for CK-MB were conjugated to gold urchin AuNPs, those for cTnT to AuNPs, and GPBB to AgNPs to provide visible purple, red, and gold color spots respectively.*

non-coated paper or paper modified with one of the other three reagents. The APTES and AuNPs supported the correct orientation of the immobilized enzyme which enhanced color intensity and provided uniform distribution of color (Figure 3).

Multiplex PADs can be developed to profile lipid analytes in a flower-shaped format (Figure 4) [https://doi.org/10.1016/j.snb.2019.01.064]. Linearity was maintained in the range of 50-400 mg/dL for total cholesterol (Tc), 70-300 mg/dL for triglycerides (TGL), and 70-400 mg/dL for LDL. The results of the PADs agreed with results from Roche-COBAS C11 autoanalyzer for Tc, LDL, HDL, and TGL: the Pearson correlation coefficients (R) were 0.90, 0.97, 0.99, and 0.99, respectively. Thus, modifications on the paper substrate can improve binding efficiency of biomolecules and the sensitivity and specificity of POC tests.

Fabrication of 3D PADs

Challenges in 3D PADs include alignment, bonding steps, and delay of liquid flows. To minimize these challenges, Kwak and coworkers [https://doi.org/10.1039/C8AY01318G] used a vacuum-driven polydimethylsiloxane (PDMS) stamping method and created a hydrophobic barrier in the hydrophilic paper for fabrication of 3D PADs. They combined it with a cut- and insert-method which used strong physical contact to securely connect two or more layers of the device. Together, 3D PAD prototypes based on PDMS stamping and cut- and insert-method provided improved all-directional and long-distance 3D-liquid flow versus a plasma-enhanced PDMS bonding device. Different configurations of five dog-bone shaped channels showed tight and mechanically interlocked junctions between vertical and horizontal regions and provided efficient transfer of blue and yellow dye solutions. Importantly, a distorted device caused by wetting or overloading of sample maintained tight bindings.

Kwak and coworkers developed a device to simultaneously measure glucose, nitrite and pH by using five PADs and the cut- and insert-method (Figure 6) [https://doi.org/10.1039/C8AY01318G]. The glucose and nitrite assays used the Benedict reagent (11 945, Fluka) and nitrite detection kit (API®), respectively. The

Figure 3

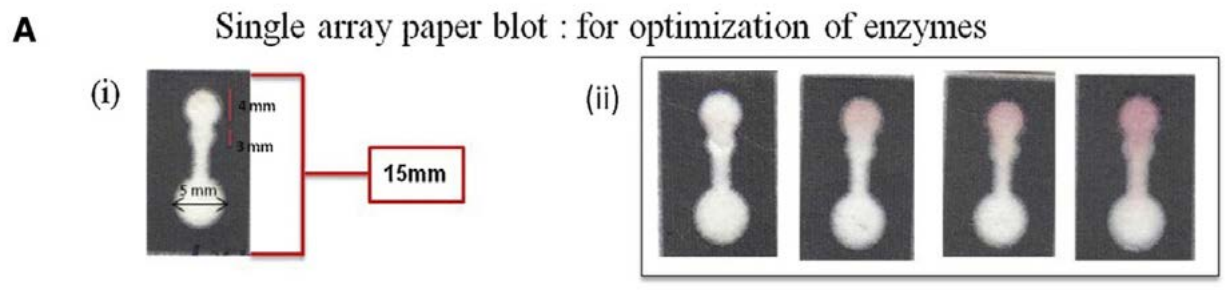


Fig. 3: *Comparison of color intensity and distribution on PADs which had been modified with chitosan, AuNPs, 3-aminopropyltriethoxysilane (APTES) and AuNPs with APTES. Reproduced with permission https://doi.org/10.1016/j.snb.2019.01.064*

Figure 4

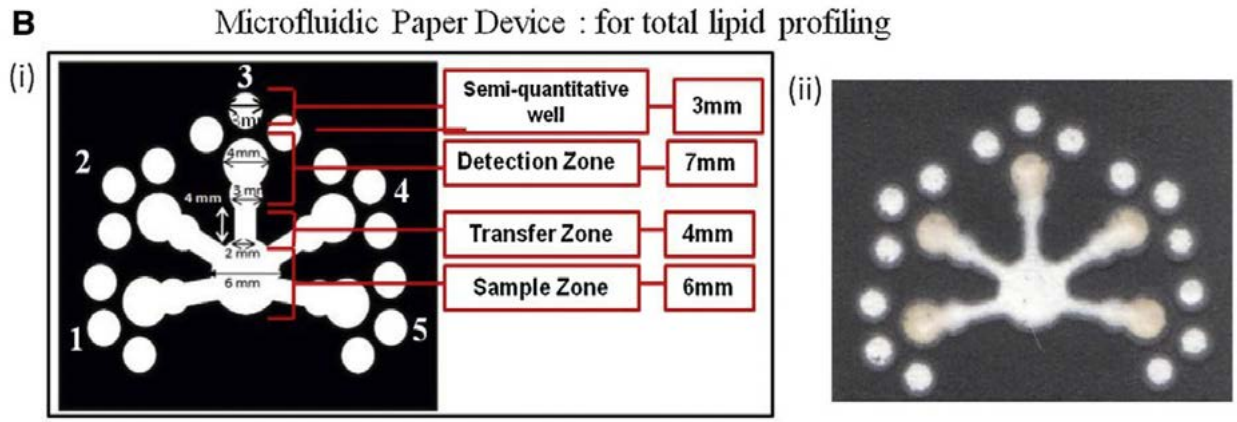


Fig. 4: *Schematic illustration of flower-shaped channels for multiplex analysis in paper-based devices.*

Benedict reagent changed color from blue to yellow and the API® changed color from blue to purple.

A digital camera captured the blue intensity of the pH for quantitative purposes. All measurements showed good linearity (R2>0.96). Minimum LODs were 0.25ppm for nitrite, 0.31 mg/dL for glucose and pH1 for pH measurement. Since the red, green, and blue intensity still varied, further improvements on method accuracy are encouraged and warranted.

Timed fluid control

Automation of immunoassay-based PADs can be enhanced by timed fluid control (TFC). These three methods can achieve TFC:

- Modifying the channel geometry to control flow rate
- Having specific chemicals (eg, sugar) in the flow path, changing the viscosity and creating a programable delay in flow
- Using a mechanical valve to turn reaction on or off. It usually is combined with programmed channel geometry

Wang and coworkers integrated a magnetically actuated value into a CLIA-based PAD for rapid determination of three tumor markers (Figure 7) [https://doi.org/10.1016/j.snb.2017.07.192]. The PAD was fabricated with photolithography on Whatman chromatographic paper grade 1. Immobilization of the capture antibodies for CEA, alpha fetoprotein (AFP), and CA-125 was achieved by coating with chitosan and crosslinking with glutaraldehyde. The time controller used an electric switch comprised of two moveable conductive iron bands connected by the fluid flow. When the circuit closed, the magnetic value was actuated which began the HRP reaction. The method showed good correlation with a standard ELISA: Pearson correlation coefficient equals 0.98. This immunoassay-based PAD was completed in 16 min, which is much shorter than the conventional CLIA completion time of 2 hr.

In summary, advantages of these devices include simplicity, portability, lack of sophisticated instruments, user-friendly, and low cost which supports their use in POC testing. Challenges may include stability of reagents, and storage of PADs, samples, and reagents to maintain activity. Research of the sample matrix for the proposed LFA can include sample preparation, storage conditions, effect of analyte on flow through PAD, and cross reactivity. Continued development and improvements will broaden the use of PADs in the POC setting for determination of biomarkers.

Figure 5

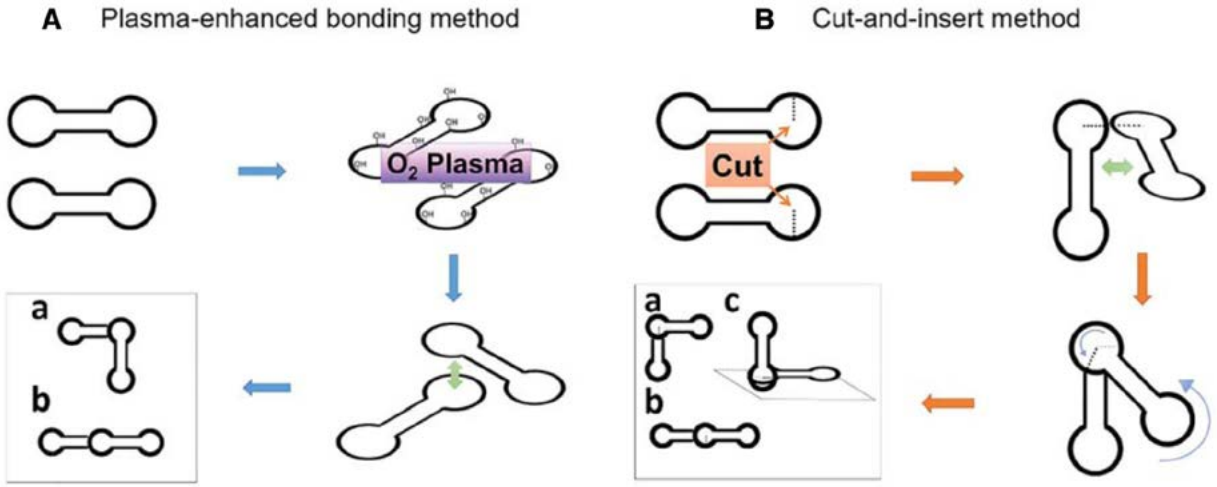


Fig. 5: *Schematic illustrations for construction methods of microPADs devices. (A) Plasma-enhanced bonding method. (B) Cut- and insert-method.*

Figure 6

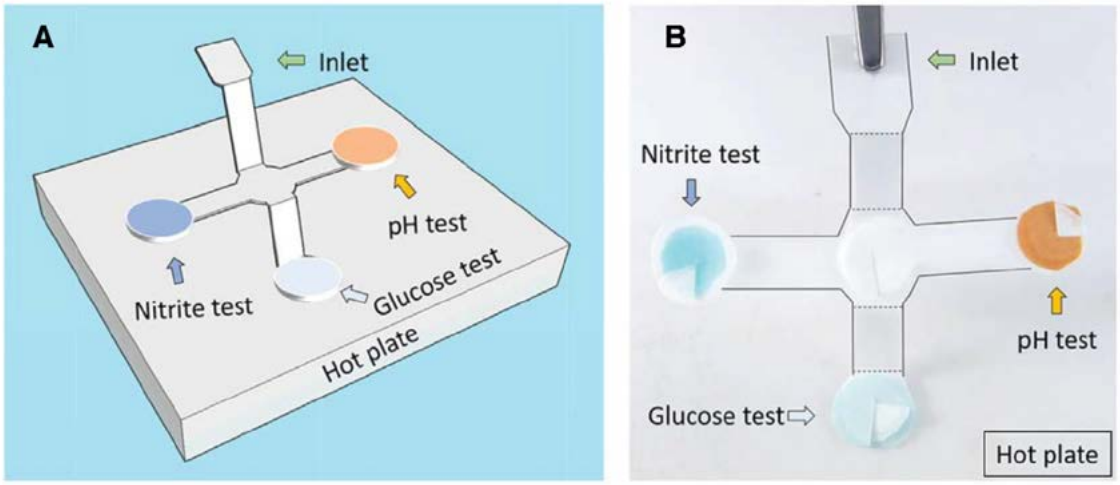


Fig. 6: *Schematic illustration of experimental set-up with three parallel colorimetric sensing stations. (A) Schematic diagram of parallel colorimetric sensing platform. (B) Picture of actual parallel colorimetric sensing μ-PADs. The cut-and insert-method connected each circular area in the μ-PADs for nitrite, glucose, and pH to the outlet of non-treated μ-PADs. For better visualization, black lines indicate the edge of the μ-PADs and dashed lines designate the bended areas. Reproduced with permission from https://doi.org/10.1039/C8AY01318G.*

Figure 7

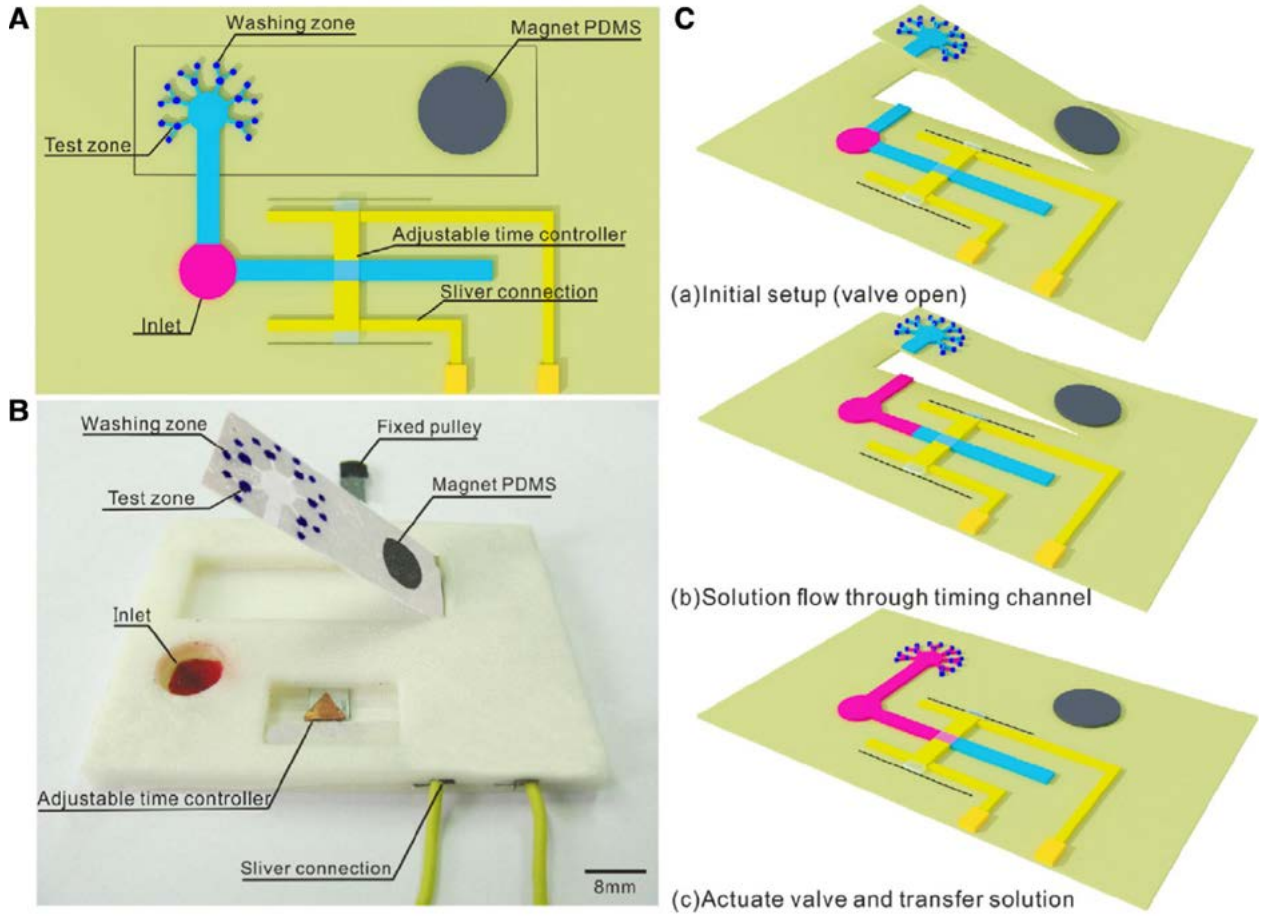


Fig. 7: *Schematic illustration of magnetic valve with controllable timing capability in PAD. (A) PAD with an electromagnetic valve, timing channel, and a moveable timing unit. (B) Photograph of PAD with an external 3D-printed plastic cover. (C) Schematic diagram for triggering mechanism of electromagnetic valve.*

BACK TO CONTENTS

Diagnostic Ability of Salivary Matrix Metalloproteinase-9 Lateral Flow Test Point-of-Care Test for Periodontitis

Kim H-D, Lee C-S, Cho H-J *et al.*

Article

Periodontal disease affects >700 million people globally. The ability to detect specific changes in the saliva or oral cavity that can predict periodontitis would allow early intervention, i.e., before clinical examination observes increased probing depth, reduced clinical attachment of gums to teeth, and bone destruction. Gingivitis, which is characterized by swelling, redness, and bleeding of the gums, often proceeds to periodontitis which involves the periodontal ligament and alveolar bone [https://doi.org/10.1186/s12903-022-02185-3]. However, the current activity of periodontitis and its progression are not predicted by these clinical parameters [https://doi.org/10.1038/nrdp.2017.38]. Because periodontitis leads to irreversible destruction of periodontal tissues, tests that provide early detection of risk of periodontitis are warranted.

Changes in inflammatory biomarkers in saliva have included inflammatory mediators (eg, salivary matrix metalloproteinases (MMPs), IL-1β, IL-6) and periodontopathic bacteria. Patients with chronic or advanced periodontal disease had higher concentrations of MMPs in saliva on average than healthy controls. MMPs are related enzymes that can degrade extracellular matrix components such as elastin, fibrinogen, collagen, laminin and proteoglycans. Extracellular matrix is maintained by the cell division of connective tissue cells and the rates of synthesis and activation of local MMPs and its tissue inhibitors (TIMPs). Saliva from patients with periodontal disease have included active MMP-3, MMP-8, and MMP-9.

The authors developed and tested a lateral flow assay based on detecting active MMP-9 in saliva of patients with periodontitis and healthy controls.

Figure 1

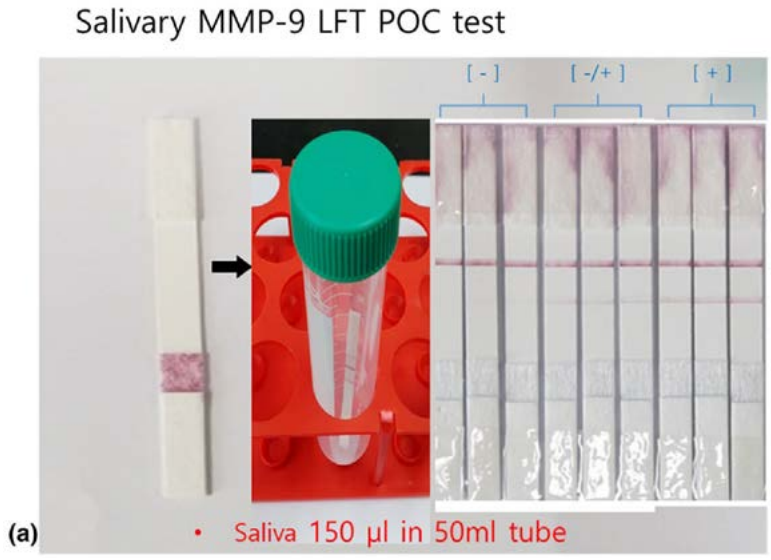


Fig. 1: *Illustration of new LFA strip, the incubation set-up, and LFA strips after procedure.*

Saliva sample preparation

Patients who had not eaten, drank, or brushed their teeth within 1 hour of collection provided unstimulated whole saliva in the morning (8 am – noon) by using the spitting method or the passive drool method into a 50 mL sterile conical tube. Saliva secretion rate was recorded. Both raw and centrifuged saliva samples were tested in the lateral flow assays (LFAs). Saliva was centrifuged for 15 min at 2600 g at 4°C and supernatant (1 mL) was aliquoted in 1.5 Eppendorf tubes and stored at -80°C.

Table 1

	Lateral flow test			
Variable	**Total (n = 137)**	**Negative (n = 46)**	**Positive (n = 91)**	**p-value**
Age, mean ± SD	48.60 ± 22.15	34.07 ± 17.13	55.95 ± 20.79	**<.001**
MMP-9, ng/ml	134.98 ± 156.64	27.77 ± 34.77	189.18 ± 166.21	**<.001**
Flow time, minute	20.46 ± 10.66	19.65 ± 11.82	20.87 ± 10.05	.530
Sex, n (%)				
Male	67 (48.9)	23 (50.0)	44 (48.4)	.855
Female	70 (51.1)	23 (50.0)	47 (51.6)	
Smoking, n (%)[a]				
No	107 (78.1)	39 (84.8)	68 (74.7)	.179
Yes	30 (21.9)	7 (15.2)	23 (25.3)	
Obesity, n (%)[b]				
No	103 (75.2)	35 (76.1)	68 (74.7)	.862
Yes	34 (24.8)	11 (23.9)	23 (25.3)	
Saliva type, n (%)				
Centrifuged	82 (59.9)	31 (67.4)	51 (56.0)	.201
Raw	55 (40.1)	15 (32.6)	40 (44.0)	

Note: **Bold denotes statistical significance at p < .05**. SD: standard deviation p-value obtained from chi-square test for categorical variables and from t test for continuous variables such as age, mmp-9 and flow time. [a]Smoking: No = never smoked, Yes = past and current smoker. [b]Obesity: No = body mass index (BMI: weight[Kg]/ height[M^2]) <25, Yes = BMI ≥25)

Table 1: *Characteristics of participants grouped by results of LFA tests.*

Consistency in sample collection, processing, and storage helped maintain reproducibility.

Concentration of MMP-9 in saliva samples was determined with a commercial ELISA kit by using diluent solution for eight 2-fold dilutions, according to manufacturer's instructions. Reproducibility was very high (Pearson r=0.99).

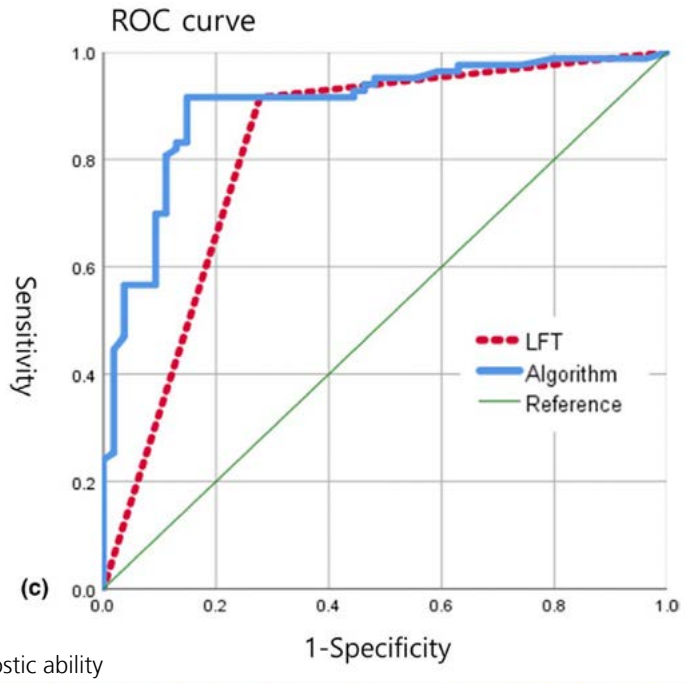
Development of LFA

The five main components of the lateral flow assay were a plastic backing, nitrocellulose membrane, a sample pad, a conjugate pad for the conjugated detection molecule, and an absorbent pad. The absorbent pad designed for oral fluid collection transports the oral fluid into the sample pad.

The LFA strip used the following components:

- 50 mL test tube for incubating LFA with 150 µL sample
- Sample pad for 150 µL of saliva
- Red conjugate pad which was sprayed with the gold nanoparticle (Au-NPs)-conjugated to anti-MMP-9 antibodies (and dried)
- The control was anti-rabbit-IgG (C line)
- Absorbent pad

Figure 2



Diagnostic ability

Type	Sensitivity/Specificity (C-statistics)	Cut-off	AUC (95% CI)	P-value
Algorithm	0.916/0.852 (0.884)	0.5885	0.900 (0.845-0.955)	<0.001
LFT	0.916/0.722 (0.819)	0.5	0.819 (0.739-0.899)	<0.001

Fig. 2: *The ROC curve for the lateral flow test (LFT) and for the algorithm that included adjustments for known risk factors of periodontitis.*

Table 2

	LFT/ Periodontitis		
	Periodontitis Negative	**Periodontitis Positive**	
Stratum	**N_{LFT}/N_{perio} (Specificity)**	**N_{LFT}/N_{perio} (Sensitivity)**	**C-statistics**
Total	39/54 (0.722)	76/83 (0. 916)	0.819
Sex			
Male	19/27 (0.704)	36/40 (0.900)	0.802
Female	20/27 (0.741)	40/43 (0.930)	0.836
Smoking[a], n (%)[a]			
No	33/46 (0.717)	55/61 (0.902)	0.809
Yes	6/8 (0.750)	21/22 (0.955)	0.852
Obesity[b], n (%)[b]			
No	30/42 (0.714)	56/61 (0.918)	0.816
Yes	9/12 (0.750)	20/22 (0.909)	0.829
Saliva type, n (%)			
Centrifuged	25/38 (0.658)	38/44 (0.864)	0.761
Raw	14/16 (0.875)	38/39 (0.974)	0.925

Note: **Bold denotes statistical significance at p < .05**. [a]Smoking: No = never smoked, Yes = past and current smoker. [b]Obesity: No = body mass index (BMI: weight[KgJ/ height[M?]) <25, Yes = BMI ≥25)

Table 2: *Effect of sex, smoking, obesity, and saliva type on risk for periodontitis.*

The authors added the LFA strip into the tube with 150 µL of raw or centrifuged salvia and incubated at room temperature (Figure 1) until the control line showed a thick red band and the colors of the conjugate and absorbent pads changed to white and red, respectively. It completed in approx. 20 min.

Concentration of MMP-9 in saliva samples was determined with a commercial ELISA kit by using diluent solution for eight 2-fold dilutions, according to manufacturer's instructions. Reproducibility was very high (Pearson r=0.99).

Association of positive salivary MMP-9-based LFA with periodontitis

Comparison of the characteristics of the patients with their results in the MMP-9-based LFA revealed that only older age (≥35) and higher concentration of MMP-9 were significantly associated with positive LFA results (Table 1).

These results raised the possibility that confounders such as sex, smoking, obesity, and type of saliva sample had less effect on the LFA results than MMP-9 but may still affect the sensitivity and specificity of the assay. Thus, the effect of each confounder on the negative MMP9-based LFA of total negative periodontitis (specificity) and on the positive MMP-9-based LFA of total positive periodontitis patients are shown in Table 2.

The MMP-9-based LFA displayed a screening ability for periodontitis (area under the curve (AUC) of 0.82 and a concordance (C-statistic) of 0.82). Its sensitivity was 0.92 and specificity of 0.72. Stratification by the type of saliva sample showed that raw saliva samples showed the highest diagnostic ability of LFA-POC test for periodontitis. The sensitivity was 0.97 and specificity of 0.88 with a C-statistic of 0.93. The potential confounding factors of obesity, sex and smoking did not significantly alter the screening ability of LFA-POC test.

LFA and older age was significantly associated with periodontitis by logistic regression analysis, as shown in Table 3.

To maximize the ability of the LFA to predict periodontitis based on MMP-9 positivity in saliva, an algorithm was developed that took into account the results of the MMP-9 results and the well-known risk factors by using multivariable logistic regression models. Effect modification was based on stratified associations according to sex, age groups (less than 35, 35 and older), obesity (yes, no) and type of saliva (raw, centrifuged). The resulting algorithm is shown below:

Algorithm score =

-3.675
+ 2.877*LFT
+ 0.034*age
+ 0.121*sex
+ 0.372*smoking
+ 0.192*obesity

Assuming the algorithm has a cut-off value of 0.589, the screening ability of the LFA-POC improved with the algorithm: the AUC was 0.9 and C-statistic of 0.88 (Figure 2). Sensitivity was 0.92 and specificity was 0.85.

The screening ability of the algorithm and the LFA (Figure 2) indicates that both analyses have a significant diagnostic ability for periodontitis (p<0.001). The algorithm improved the specificity of the results of the MMP9-based LFA. Both the algorithm and the LFA results showed a sensitivity of 92%, suggesting that potentially 8 of 100 patients with periodontitis would be missed. Alternatively, one or more of these misclassified patients may have a disease associated with periodontitis such as rheumatoid arthritis, diabetes, or cardiovascular disease.

Table 3

	Periodontitis			
Variables	**Beta**	**SE**	**Odds ratio (95% CI)**	**p-value**
LFT	2.877	0.523	**17.764 (6.375-49.497)**	**<.001**
Age	0.034	0.012	**1.035 (1.011-1.059)**	**.004**
Sex	0.121	0.516	1.129 (0.411-3.101)	.814
Smoking	0.372	0.694	1.451 (0.373-5.651)	.592
Obesity	0.192	0.608	1.211 (0.368-3.987)	.753

Abbreviations: CI, confidence interval; SE, standard error. **Bold denotes the statistical significance at p < .05**.

Table 3: *Association of lateral flow test (LFT) with periodontitis by logistic regression analysis (n = 96)*

Regarding sample choice, the raw saliva samples (n=37) compared to the total sample set exhibited higher diagnostic ability: sensitivity of 97% vs 92%, specificity of 88% vs 72%, and a C-statistic of 93% vs 82%. The lack of processing step (centrifugation) reduced the preparation time although raw saliva samples have a higher flow time.

Regarding analyte choice and preparation, this LFA used the humanized monoclonal antibody GS-5745 antibody that binds to MMP-9 distal to the active site, specifically at the junction between the propeptide and the catalytic domains. It does not recognize the active MMP-9. Further studies are warranted to assess the diagnostic ability, predictive ability, and prognostic ability of LFA measuring active MMP-9 vs all MMP-9 vs propeptide MMP-9.

Several commercial LFAs measuring MMP-8 have a lower diagnostic ability for periodontitis: the authors reported that Periosafe had a specificity of 81% and a sensitivity of 40% among 130 adults (95 patients with periodontitis) whereas Periomarker® had a sensitivity of 81% and specificity of 46% among 188 adults (139 with periodontitis). A POC test for active MMP-8 appeared to have a higher sensitivity (64%-100%) and specificity of 65%-100%. Further research on active vs propeptide of MMP-8 to assess their diagnostic, predictive, and prognostic abilities in periodontitis patients and healthy controls is also warranted.

BACK TO CONTENTS

Diagnostic Accuracy of Serological Tests and Kinetics of Severe Acute Respiratory Syndrome Coronavirus 2 Antibody: A Systematic Review and Meta-Analysis

Mekonnen D, Mengist H.M., Derbie A, *et al.*

Article

This study aimed to assess the accuracy of three methods for determining antibody responses to human samples with severe acute respiratory syndrome caused by the coronavirus-2 (SARS-CoV-2). The three diagnostic test methods analyzed here included enzyme-linked immunosorbent assay (ELISA), chemiluminescence assay (CLIA), and lateral flow immunoassay (LFA).

After systemically reviewing 228 articles, they found 24 articles that met the criteria for meta-analysis which are listed in Table 1.

Risk of Bias and Applicability Concerns

They rated each study for the risk of bias and applicability concerns according to the Preferred Reporting Items for Systematic Reviews and Meta–Analyses guideline. Briefly, two researchers independently used QUADAS-2 [https://pubmed.ncbi.nlm.nih.gov/22007046/] to assess quality of articles on diagnostic test accuracy. As shown in Figure 1, most articles did not show a definite high risk of bias (all < 15%). Studies with a low risk of bias ranged from 30% to 78%. The applicability concerns in vast majority of studies were low.

Figure 1

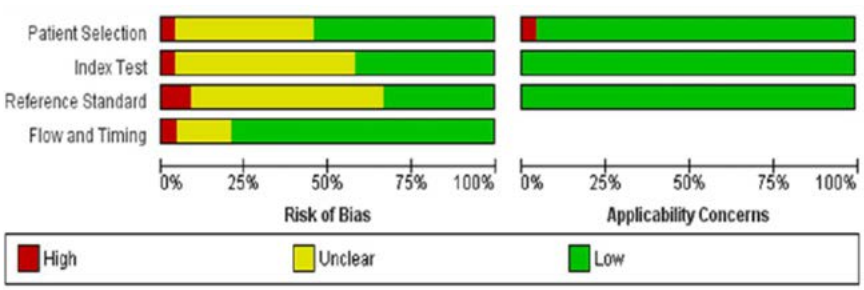


Fig. 1: *Risk of bias and applicability concerns across included studies. Two authors used QUADAS-2 to provide judgements for each included study.*

Sensitivity and Specificity

The Overall Diagnostic Test Accuracy (DTA) was calculated for the assay type (LFA, ELISA, CLIA), antibody, and antigen by meta-analysis. The number of CLIA tests (n=21), ELISA (n=37) and LFA (n=34) were relatively small in this early meta-analysis of development of SARS-CoV-2 diagnostic tests.

CLIA

The sensitivity and specificity of the CLIA are shown in a forest plot (Figure 2). The sensitivity of the CLIA ranged from 0.57 to 1.00 for the different articles and the specificity ranged from 0.80 to 1.00 (Figure 2). The CLIA summary sensitivity was calculated as 92% with a 95% confidence interval (CI): 86%-95%. Similarly, the CLIA summary specificity was 99% (95% CI: 97%-99%).

ELISA

The sensitivity and specificity of the ELISA are shown in a forest plot (Figure 3). The ELISA sensitivity ranged from 0.57 to 1.00 for the different articles and the specificity ranged from 0.80 to 1.00 (Figure 3). The ELISA had a summary sensitivity of 86% (CI: 82%-89%). The ELISA summary specificity was 76% (95% CI: 58%-88%).

ELISA had the lowest DTA of the three assays. Improved summary sensitivity was observed in IgA antibody-based ELISA (91% CI: 81-96%; n=5) and total antibody-based ELISA (94% CI: 90-97%; n=9) although these results need confirmation due to using only a few samples.

Table 1. The 24 articles included in the meta-analysis

References	Assay	Company	Antibody types	Antigen
Adams et al https://doi.org/10.1101/2020.04.29.20082099	ELISA	In-house	IgM/IgG	S
Adams et al https://doi.org/10.1101/2020.04.29.20082099	LFA	9 Anonymous companies	IgM/IgG	S
Adams et al https://doi.org/10.1101/2020.04.15.20066407	ELISA	Mologic's IgG ELISA	IgG	N+S
Bryan et al https://doi.org/10.1128/jcm. 00941-20	CLIA	Abbott	IgG	N
Burbelo et al https://doi.org/ 10.1093/infdis/jiaa273	LIPS	Twist Biosciences	Total Ab	N, S
Cai et al https:// doi.org/10.1093/infdis/jiaa243	CLIA	Sangon Biotech Co	IgM, IgG, IgM/IgG	S
Freeman et al https://doi.org/10.1101/2020.04.24.057323	ELISA	Thermo Fischer	IgM, IgG, Total Ab	S
GeurtsvanKessel et al https://doi.org/10.1101/ 2020.04.23.20077156	LFA	Cellex Inc.	IgG	N+S
GeurtsvanKessel et al https://doi.org/10.1101/ 2020.04.23.20077156	CLIA	DiaSorin Liaison	Total Ab	S
GeurtsvanKessel et al https://doi.org/10.1101/ 2020.04.23.20077156	ELISA	EUROIMMUN Medizinische	IgG, IgA	S
GeurtsvanKessel et al https://doi.org/10.1101/ 2020.04.23.20077156	LFA	InTec	IgM, IgG	N
GeurtsvanKessel et al https://doi.org/10.1101/ 2020.04.23.20077156	LFA	Orient Gene	IgM, IgG	N+S
GeurtsvanKessel et al https://doi.org/10.1101/ 2020.04.23.20077156	ELISA	Wantai Biological Pharmacy	IgM, Total Ab	RBD
Guo et al https://pubmed.ncbi.nlm.nih.gov/32198501/	ELISA	In-house	IgM, IgA, IgG	N
Infantino et al https://pubmed.ncbi.nlm.nih.gov/26404177/	CLIA	YHLO Biotech	IgM, IgG	No data
Lassauniere et al https:// doi.org/10.1101/2020.04.09.20056325	LFA	Acro Biotech, AllTest Biotech, Artron Laboratories, AutoBio Diagnostics, CTK Biotech, Dynamiker Biotechnology	IgM/IgG	No data
Lassauniere et al https:// doi.org/10.1101/2020.04.09.20056325	ELISA	Wantai Biological Pharmacy	IgG, IgA, Total Ab	RBD
Lin et al https://doi.org/10.1007/s10096-020-03978-6	CLIA	Darui Biotech	IgM, IgG, IgM/IgG	N
Liu Wet al https://doi.org/10.1128/jcm.00461-20	ELISA	Hotgen, Beijing	IgM, IgG, IgM/IgG	S, N
Liu Ret al https://doi. org/10.1101/2020.03.28.20045765	ELISA	YHLO Biotech	IgM	No data
Lou et al https://doi.org/10.1183/ 13993003.00763-2020	LFA	Wantai Biological Pharmacy	IgM, IgG	RBD
Lou et al https://doi.org/10.1183/ 13993003.00763-2020	LFA, ELISA	Wantai Biological Pharmacy	IgG	N
Lou et al https://doi.org/10.1183/ 13993003.00763-2020	ELISA	Wantai Biological Pharmacy	IgM, IgM/IgG, Total Ab	RBD
Lou et al https://doi.org/10.1183/ 13993003.00763-2020	CLIA	Xiamen InnoDx Biotech	IgM	RBD

Table 1 continued

References	Assay	Company	Antibody types	Antigen
Lou et al https://doi.org/10.1183/ 13993003.00763-2020	CLIA	Xiamen InnoDx Biotech	IgM, IgG	N+S
Ma et al https:// doi.org/10.1038/s41423-020-0474-z	CLIA	In-house	IgM, IgG, IgA & their combination	RBD
Meyer et al https://doi.org/10.1016/j.cmi.2020.06.024	ELISA	EUROIMMUN Medizinische	IgA, IgG	S
Okba et al https://doi.org/ 10.3201/eid2607.200841	ELISA	In-house	IgM, IgG, IgM/IgG	S
Pan et al https://doi.org/10.1016/j. jinf.2020.03.051	LFA	Zhu Hai Liv Zon Diagnostics	IgM, IgG, IgM/IgG	No data
Perez-Garcia et al https://doi.org/10.1101/2020.04. 11.20062158	LFA	AllTest Biotech, Hangzhou	IgM, IgG, IgM/IgG	No data
Whitman et al https://doi.org/10.1101/2020.04.25. 20074856	LFA	BioMedomi cs	IgM, IgG, IgM/IgG	RBD
Whitman et al https://doi.org/10.1101/2020.04.25. 20074856	LFA	Bioperfectus, Sure	IgM, IgG, IgM/IgG	N+S
Whitman et al https://doi.org/10.1101/2020.04.25. 20074856	LFA	DecomBio, DeepBlue	IgM, IgG, IgM/IgG	No data
Whitman et al https://doi.org/10.1101/2020.04.25. 20074856	ELISA	Epitope	IgM, IgG, IgM/IgG	N
Whitman et al https://doi.org/10.1101/2020.04.25. 20074856	ELISA	In-house	IgM, IgG, IgM/IgG	RBD
Whitman et al https://doi.org/10.1101/2020.04.25. 20074856	LFA	Innovita	IgM, IgG, IgM/IgG	N+S
Whitman et al https://doi.org/10.1101/2020.04.25. 20074856	LFA	Premier, UCP, VivaChek. Wondfo	IgM, IgG, IgM/IgG	No data
Xiang et al https://doi.org/10.1101/ 2020.02.27.20028787	LFA	Zhu Hai Liv Zon Diagnostics	IgM, IgG, IgM/ IgG, Total Ab	No data
Zhang et al https://doi. org/10.1101/2020.03.17.20036954	LFA	In-house	IgM/IgG	RBD
Zhao J et al https://doi.org/10.1093/cid/ciaa344	ELISA	Wantai Biological Pharmacy	IgM, Total Ab	RBD
Zhao J et al https://doi.org/10.1093/cid/ciaa344	ELISA	Wantai Biological Pharmacy	IgG	N
Zhao R et al https://doi.org/10.1101/ 2020.03.26.20042184	ELISA	In-house	IgM/IgG	S
LFA, lateral flow immunoassay; N, nuclear protein; RBD, receptor-binding domain; S, spike protein,				

LFA

Figure 4 shows the sensitivity and specificity of the LFA in a forest plot. The LFIA sensitivity ranged from 0.22 to 1.00 for the different articles and the specificity ranged from 0.73 to 1.00 (Figure 4). The LFA summary sensitivity was 78% (95% CI: 71%-83%). Its summary specificity was 98% (95% CI: 96%-99%).

Effect of Antibodies and Antigens

Each subgroup of antibody and antigen used a small number of studies in CLIA,

Figure 2

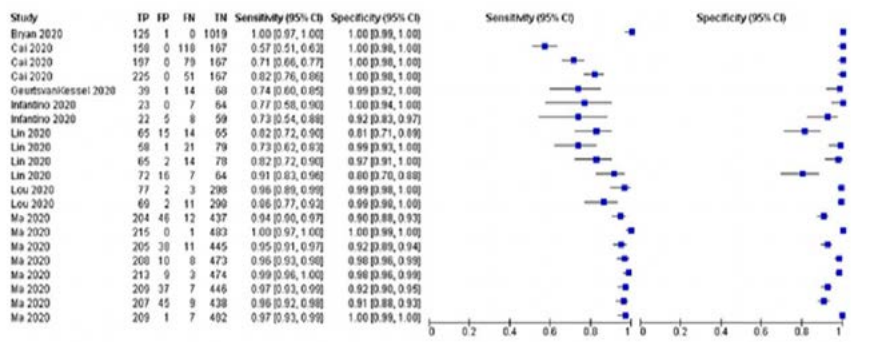


Fig. 2: *Forest plot of CLIA sensitivity and specificity. TP, true positive; FP, false positive; TN, true negative; FN, false negative.*

ELISA, and LFIA , ranging from 4-9, 4 to 15, and 4 to 20, respectively. Thus, conclusions should be made with caution.

CLIA

Although tested in a few samples, the CLIA-IgM (84%, 95% CI: 67-93%) had a modestly lower sensitivity than the CLIA-IgG (92%, 95% CI 71%-98%) and CLIA-IgM/IgG assays (92%, 95% CI: 85%-96%). The differences did not reach statistical significance.

Regarding antigens, the CLIA that used the RBD of spike protein showed higher sensitivity (96%, 95% CI: 94%-98%) than either nucleocapsid (N) and spike protein (S). The specificity of all three assays ranged from 97% to 99%.

ELISA

The sensitivity of ELISA modestly varied with antibody type, ranging from 79% (95% CI:73% -85%) for IgG to 94% (95% CI: 90%-97%) for total antibody (tAb). The rankings for higher sensitivity to lower sensitivity based on antibody types were tAb> IgA> IgM/IgG> IgM> IgG, although the differences did not reach statistical significance.

The ELISA based on RBD showed higher sensitivity (92%, 95% CI: 86%-96%) than the other tested antigens: S (87%, 95% CI: 81%-91%) and N (81%, 95% CI: 72%-88%). The differences did not reach significance.

Regardless of antibody type or antigen, the specificity of the ELISA ranged from 97% to 100%.

LFA

The overall diagnostic accuracy profile of LFIA (n=34 tests) included a sensitivity of 78% (95% CI: 71%-83%) and a specificity of 98% (95% CI: 96%-99%). The IgM-based LFA had a sensitivity of 82% (95% CI: 67%-98%) whereas IgG and IgM/G based assay were modestly lower at 72% (95% CI: 50%-88%) and 77% (95% CI: 69%-84%), respectively.

LFA assays using both S and N proteins (n=4) increased the sensitivity from 78% (95% CI: 71%-83%) to 88% (95% CI: 85%-91%) although these tests displayed lower specificity at 96% (95%: 78%-99.5%).

Receiver-Operative Characteristic (ROC) Curves

The ROC illustrates both the sensitivity and specificity of each individual assay. According to convention, a summary point in the upper left corner indicates the test or parameter that supports a high diagnostic test accuracy of the tested samples.

The whole dataset grouped by type of assay, antibody or antigen was analyzed by hierarchical summary receiver-operative characteristics (HSROC) (Figure 5). Figure 5 shows that the summary point of the sensitivity-specificity HSROC curves was higher in CLIA than ELISA and LFA. These data illustrate the overall diagnostic accuracy profile of CLIA with sensitivity of 92% (CI:86%-95%) and specificity of 99% (95% CI: 97%-99%). In comparison, the summary point on the HSROC curves for ELISA and LFIA were lower.

Figure 3

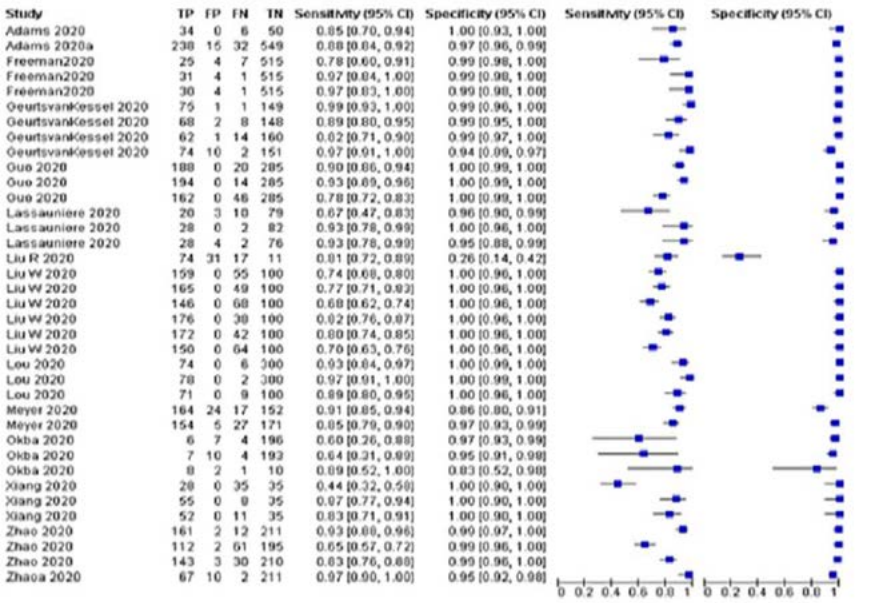


Fig. 3: *Forest plot of ELISA sensitivity and specificity. TP, true positive; FP, false positive; TN, true negative; FN, false negative.*

Figure 4

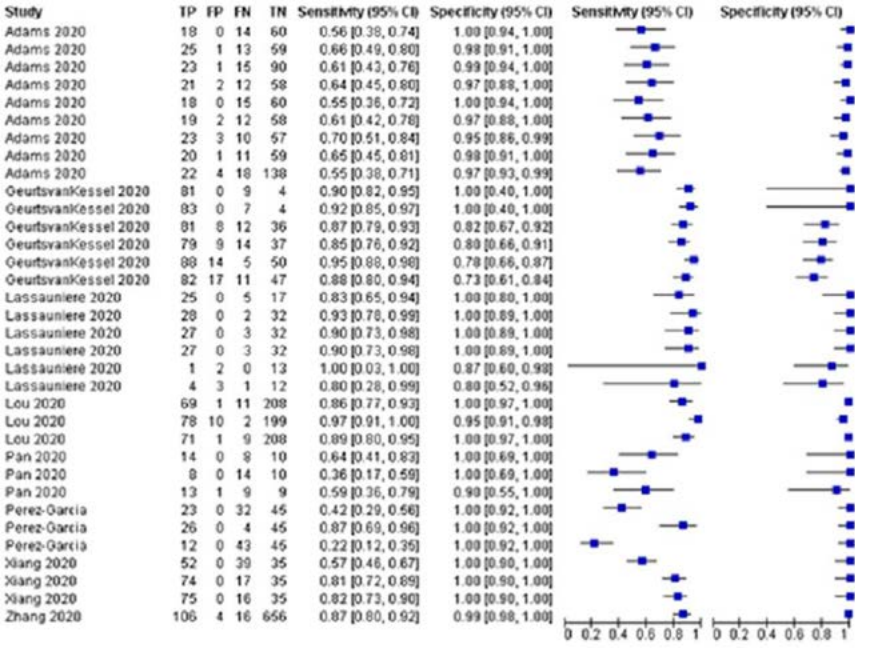


Fig. 4: *Forest plot of LFIA sensitivity and specificity. TP, true positive; FP, false positive; TN, true negative; FN, false negative.*

Figure 6 shows that the summary point of the sensitivity-specificity HSROC curves for antibody types was higher in IgA and total Antibody-based assays than IgG, IgM or IgM/IgG -based assays.

The summary point of the sensitivity-specificity HSROC curves for antibody types was higher in IgA and total Antibody-based assays than IgG, IgM or IgM/IgG -based assays.

The summary point of the sensitivity-specificity HSROC curves for types of antigens was higher in RBD than for other SARS-CoV-2 antigens, including S, N, N + S, and undisclosed antigen.

Kinetics of anti-SARS-CoV-2 antibodies

Different studies grouped days post-symptom onset (DPSO) differently, including [<14 days and >14 days], [1-7, 8-14, ≥15 days], or [1-10, 11-15, 16-20, and ≥21 days]. Because more tests were provided using the latter DPSO categories, the authors used this set. Most longitudinal samples suggested antibody positivity increasing between the 1-10 DPSO group and 11-15 DPSO group. In some cases, antibody positivity modestly declined from the 11-15 DPSO group to the 16-20 DPSO group which is consistent with earlier reports [https://doi.org/10.1056/NEJMe2028079].

IgG positivity arose at similar DPSO as IgM positivity in the DPSO groups. Although classically IgM positivity is considered to arise earlier than IgG during an infection, the long incubation time of SARS-CoV-2 (1 to 14 days) and the higher affinity (and easier detection) of IgG may help explain these observations.

CLIA-based assays detected the SARS-CoV-2 antibody in the 11-15 DPSO group with a >97% sensitivity. The 1-10 DPSO group showed a higher rate of anti-RBD positivity (>55%) and anti-N antibodies (>50%) than other antigens, which raises the question whether RBD is more immunogenic than other antigens. Due to the small number of samples and the development of several variants, further investigation of the kinetics is warranted in larger studies.

Conclusion

Their meta-analysis suggests that of the reported diagnostic tests, the CLIA exhibited the highest DTA, especially in assays targeting the RBD antigen. The authors concluded that both ELISAs and CLIA showed better accuracy but they recommended

Figure 5

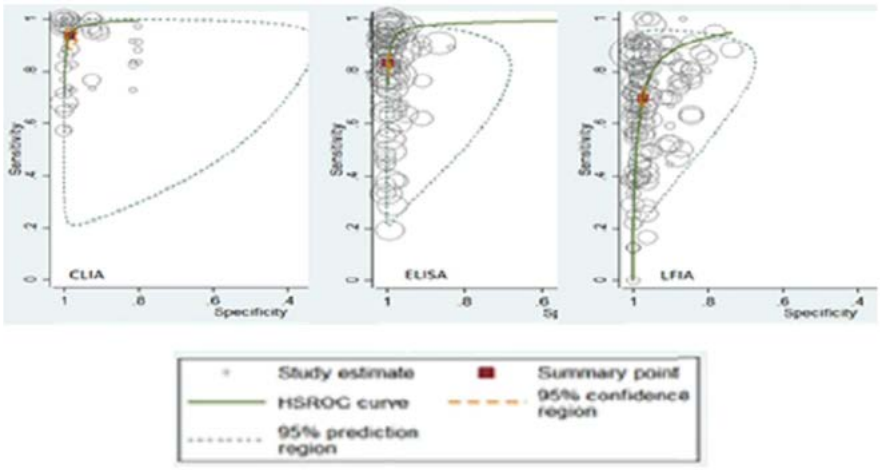


Fig. 5: *ROC curve for CLIA, ELISA, and LFIA.*

Figure 6

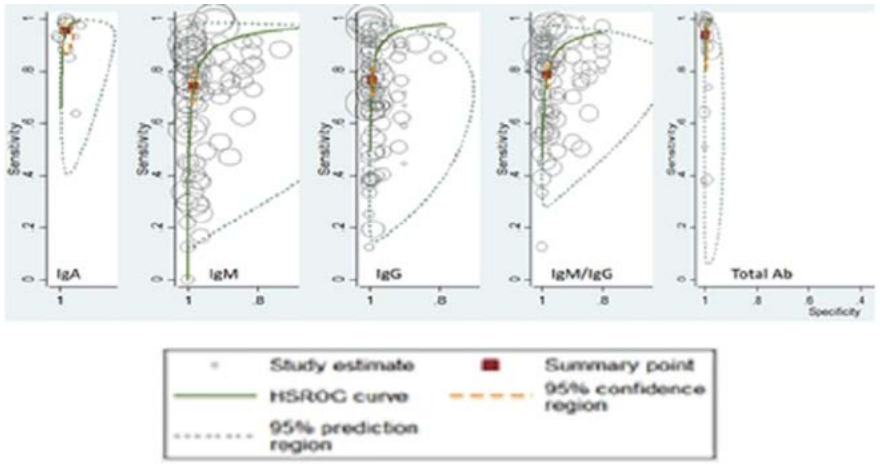


Fig. 6: *ROC curve for assays using different types of antibodies.*

Figure 7

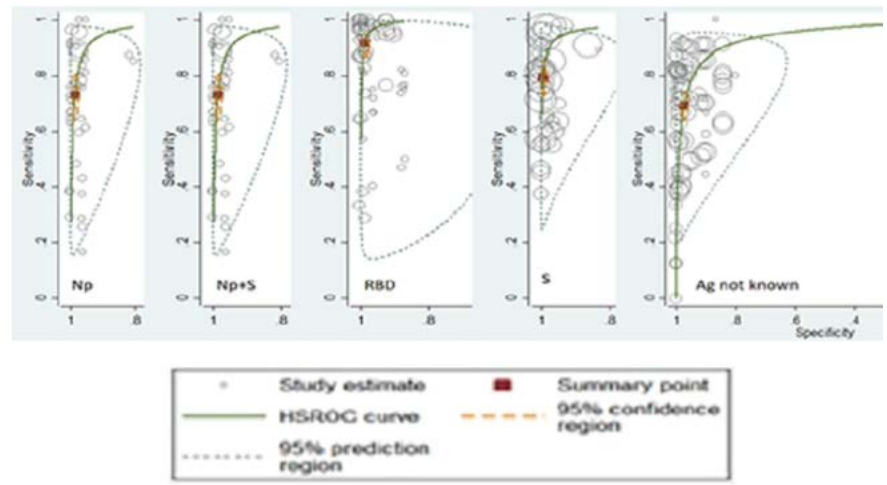


Fig. 7: *ROC curve for different types of SARS-CoV-2 antigens. Np, nucleocapsid protein; RBD, receptor-binding domain; S, spike protein; Ag not known, antigen not disclosed in articles.*

caution in using the described LFAs. However, due to the small number of studies and the continued evolving of Point of Care Tests, these conclusions should be interpreted with caution.

Figure 8

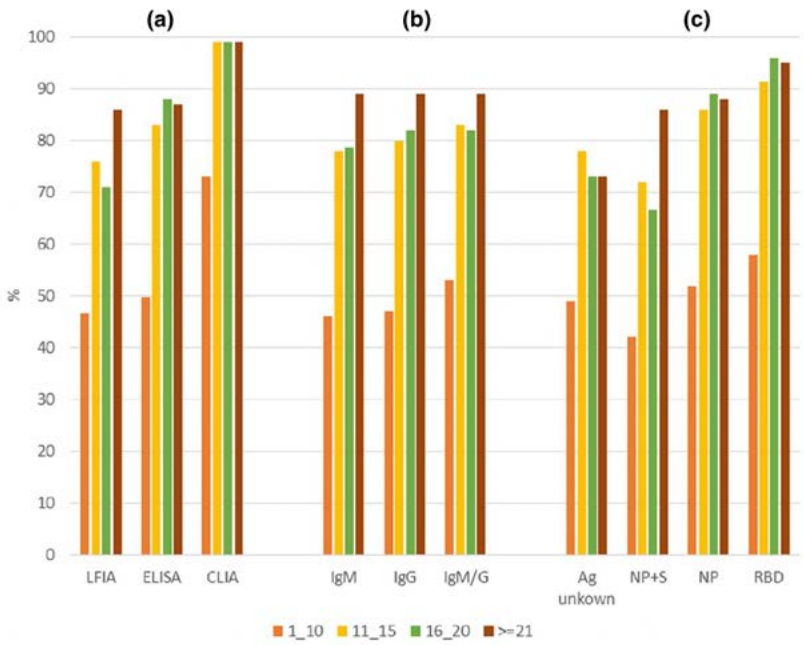


Fig. 8: *Kinetics of antibody detection as influenced by type of assay, antibody, and antigen.*

BACK TO CONTENTS

Rapid Diagnostic Testing for SARS–CoV–2: Validation and Comparison of Three Point–of–Care Antibody Tests

Strand R, Thelaus L, Fernström N, *et al*.

Article

Rapid diagnostics at Point-of-Care (POC) facilities can help physicians make decisions about disease management. Several POC tests in the lateral flow immunoassay (LFA) format are commercially available for detection of antibody (IgM and IgG) against SARS-COV-2 in whole blood, plasma, and serum: results are provided in 15 min. However, reports of the sensitivity of various tests can vary from 39% to >99%. Strand et al [https://onlinelibrary.wiley.com/doi/pdf/10.1002/jmv.26913] assessed the validity, sensitivity and specificity of three commercially available tests:

- J-test corresponds to the SARS–CoV–2 immunoglobulin (Ig) G/IgM Antibody test (Colloidal Gold) from Joinstar Biomedical Technology Co.;
- N-test corresponds to the COVID–19 IgG/IgM Rapid Test Cassette (Whole Blood/Serum/Plasma) from Noviral;
- Z-test corresponds to the ZetaGene COVID–19 rapid IgM/IgG test from ZetaGene Ltd.

The reference method for confirming an infection is reverse transcription-polymerase chain reaction (RT- PCR) testing for SARS-CoV-2 in patient samples. While the standard method, RT-PCR may not be sufficiently sensitive to detect SARS-CoV-2 during the early incubation period in some patients. Many patients develop antibodies against SARS-CoV-2 by two weeks after onset of symptoms.

Data collection

Nasopharyngeal swab samples of patients suspected of SARS-CoV-2 were tested for SARS-CoV-2 by an in-house RT-PCR procedure according to the World Health Organization (WHO) guidelines with a few modifications. After ethical approval by the Swedish ethical committee and collection of informed signed consents, convalescent blood samples were collected from 47 patients who were at least 28 days after RT-PCR confirmed diagnosis. Serum was harvested and stored at -80°C. The 50 patients in the negative control group had been treated for respiratory tract infections and discharged from the hospital 4-6 week before blood and serum collection. Serum was collected between 1997 and 2007, i.e., before SARS-CoV-2.

The sample size of the J and Z-tests was 10 µL, whereas the N-test only required 5 µL, i.e., about half the sample size of the other 2 tests.

Sensitivity

The N-test exhibited the highest sensitivity for the IgG line of the 3 tests (Figure 1). N-test had a sensitivity of 96% (95% CI: 85%-99%) whereas the J-test had an observed sensitivity of 87% (95% CI: 74%–95%) and the Z-test 85% (95% CI: 72%–94%). However, the results among the 3 tests were not significantly different (p=0.4).

The N-test exhibited the highest sensitivity for the IgG line of the 3 tests (Figure 1). N-test had a sensitivity of 96% (95% CI: 85%-99%) whereas the J-test had an

Figure 1

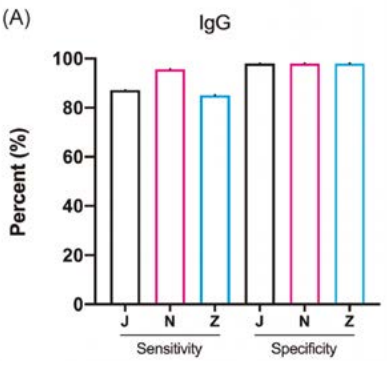


Fig. 1: *Bar chart representing sensitivity and specificity of the 3 tests in measuring SARS-CoV-2 reactive IgG in serum of RT-PCR positive patients or control patients. J-Test, SARS–CoV–2 immunoglobulin (Ig) G/IgM Antibody test (Colloidal Gold) from Joinstar Biomedical Technology Co.; N-test, COVID–19 IgG/IgM Rapid Test Cassette (Whole Blood/Serum/Plasma) from Noviral; Z-Test, ZetaGene COVID–19 rapid IgM/IgG test.*

Table 1

	IgG		IgM	
Antibody Test	**Sensitivity (%) (95% CI)**	**Specificity (%) (95% CI)**	**Sensitivity (%) (95% CI)**	**Specificity (%) (95% CI)**
N-test	96% (85%–99%)	98% (89%–100%)	67% (52%–80%)**	98% (89%–100%)
Z-test	85% (72%–94%)	98% (89%–100%)	70% (55%–83%)**	90% (78%–97%)
J-test	87% (74%–95%)	98% (89%–100%)	15% (6%–28%)	90% (78%–97%)

CI, confidence interval; .; N-Test, COVID–19 IgG/IgM Rapid Test Cassette (Whole Blood/Serum/Plasma) from Noviral; Z-Test, ZetaGene COVID–19 rapid IgM/IgG test; J-Test, SARS–CoV–2 immunoglobulin (Ig) G/IgM Antibody test (Colloidal Gold) from Joinstar Biomedical Technology Co. **p<0.001 compared to J-test.

Table 1: *Overall sensitivity and specificity of the 3 tests (N-, Z-, and J-tests) for detecting the anti-SARS-CoV-2 antibodies of IgG and IgM isotypes*

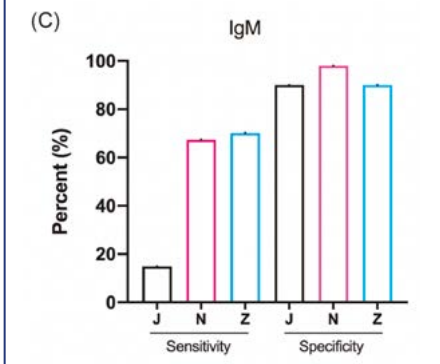


Fig. 2: *Bar chart representing sensitivity and specificity of the 3 tests in measuring SARS-CoV-2 reactive IgM in serum of RT-PCR positive patients or control patients. J-Test, SARS–CoV–2 immunoglobulin (Ig) G/IgM Antibody test (Colloidal Gold) from Joinstar Biomedical Technology Co.; N-Test, COVID–19 IgG/IgM Rapid Test Cassette (Whole Blood/Serum/Plasma) from Noviral; Z-Test, ZetaGene COVID–19 rapid IgM/IgG test.*

observed sensitivity of 87% (95% CI: 74%–95%) and the Z-test 85% (95% CI: 72%–94%). However, the results among the 3 tests were not significantly different (p=0.4). The specificity of the 3 tests for the SARS-CoV-2 antigen were the same: 98% (95% CI: 89%-100%).

The N-test and the Z-test exhibited similar sensitivities for the SARS-CoV-2 reactive IgM antibodies (Figure 2): 67% (95% CI: 52%-80%) and 70% (95% CI: 55%-83%). The sensitivity of the J-Test for the SARS-CoV-2 reactive IgM antibodies 15% (95% CI: 6%-28%) was significantly lower than those of the N-and Z-Tests (p<0.001). Specificity for the 3 tests ranged from 90% (95% CI: 78%–97%) for both the J- and the Z-tests whereas the specificity for the N-test was modestly higher at 98% (95% CI: 89%–100%) but not significantly different (p=0.2).

As a recap, Table 1 provides the overall sensitivity and specificity for detecting the anti-SARS-CoV-2 antibodies of IgG and IgM isotype.

Comparison of the positive predictive Value (PPV) with prevalence among the results of the 3 tests measuring IgG indicated no significant differences (Figure 3).

The authors stated that the many rapid diagnostic tests which detect SARS-CoV-2 antibodies can help during the process of differential diagnosis and elucidation of transmission routes. However, the tests need to be validated for sensitivity and specificity before placing them in routine clinical use. In this report, they validated 3 tests for measuring IgG specific for SARS-CoV-2.

Several additional lessons can be compiled from this study. First, seroconversion may not occur in all SARS-CoV-2 patients within 4-6 weeks as some patients may develop robust T cell responses and not seroconvert in sufficient titers to be detectable. Second, patients with severe disease often have higher titers of anti-SARS-CoV-2 antibody. About 77% of the SARS-CoV-2 patients had a mild disease course, and 5 of the 6 discordant samples had mild disease courses, suggesting that low anti-SARS-CoV-2 IgG antibody titers may explain the discordant results. Third, the lower sensitivity of the tests for anti-SARS-CoV-2 antibodies of IgM subtype may partly reflect that IgM titers are thought to decline over time.

In conclusion, this study indicated that all three tests exhibited similar sensitivity and specificity for detecting SARS-CoV-2 specific IgG antibodies in patient sera 4-6 weeks post RT-PCR confirmed diagnosis. Furthermore, the authors concluded that these 3 tests can be used in routine clinical practice due to their sufficiently high sensitivity and specificity.

Figure 3

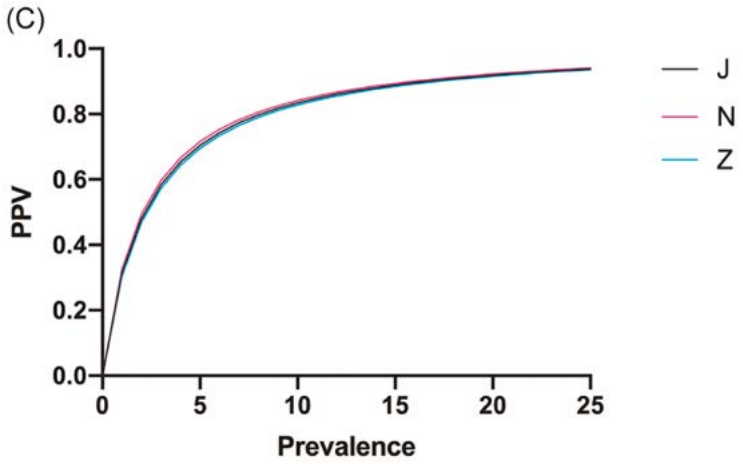


Fig. 3: *Effect of prevalence on positive predictive value (PPV) for the detection of IgG within the prevalence range of 0%–25% for the three evaluated Point–of–Care antibody tests, J, N, and Z-Tests. J-Test, SARS–CoV–2 immunoglobulin (Ig) G/IgM Antibody test (Colloidal Gold) from Joinstar Biomedical Technology Co.; N-Test, COVID–19 IgG/IgM Rapid Test Cassette (Whole Blood/Serum/Plasma) from Noviral; Z-Test, ZetaGene COVID–19 rapid IgM/IgG test.*

An Overview for the Nanoparticles-Based Quantitative Lateral Flow Assay

Wang Z, Zhao J, Xu X *et al.*

Article

Wang et al provide an overview of principles, corresponding formats, and development of LFAs for many fields, including clinical diagnosis, food safety, detection of metal ions, agriculture, and environmental monitoring. The main formats include a sandwich LFA, indirect competitive LFA, a direct competitive LFA, and a multiplexed LFA. Because of the larger size of the test antigens in clinical diagnosis of SARS-CoV-2 as well as other pathogens, LFAs have often used the sandwich LFA format. Here we focus the discussion on development of LFAs for clinical diagnosis.

Larger targets such as proteins from pathogenic viruses, bacteria, and fungi can bind to two antibodies at the same time. In a typical LFA, unlabeled antibody specific for target antigen is called the capture antibody. After the sample pad receives the positive sample solution in the T line, the positive sample percolates through the conjugation pad and solubilizes the detection antibody. The mixture migrates to the chromatographic membrane where the capture antibody binds to labeled antibody-target complex and forms a visible band at the T position, usually a red line. If the test sample is negative, no color will appear in the T line. The Control line, which also turns red, confirms the validity of the test strip. A positive result shows a colored T line and colored C line. A negative result provides a colored C line and no color in the T line.

Detection and capture biomaterials

Biomolecules are used for 3 main functions

1. Detection biomolecule specifically recognizes the target antigen
2. The capture biomolecule captures the "detection molecule-target antigen complex"
3. The signal biomolecule recognizes the "capture-target antigen-detection complex" and amplifies the signal with its substrate to a visible line.

Detection biomolecules

Examples of detection molecules include antibodies, aptamers, or protein receptors. The detection biomolecules need a strong binding affinity for the target with minimal cross reactivity to related antigens, and fast association kinetics. The exposure time between the detection biomolecule and the target antigen is 3 to 10 min in LFA which is much shorter than incubation times in ELISAs. Thus, fast association kinetics are essential.

The detection biomolecule must have sufficiently high affinity for the target antigen to maintain a stable interaction for the length of the LFA. Note that if the affinity of the detection biomolecule is not sufficiently high, it is very unlikely that optimization of its construction techniques, buffers, and physical components can overcome the insufficient affinity of the detection biomolecule.

Stability at different ion concentrations, humidity, pressure, temperature, and long storage is essential for a commercially viable detection biomolecule.

Due to their wide availability, high selectivity, and sensitivity, antibodies are widely used as detection molecules. Most antibodies also display high affinity, and usually exhibit high equilibrium association constants. Monoclonal antibodies and recombinant antibodies are often preferred over polyclonal antibodies because of the reproducibility and single specificity. Recombinant antibodies are gaining in popularity due to the potential gene instability in hybridoma cells and human reactions to mouse and / or anti-mouse antibodies. Sometimes, fragments of antibodies such as the variable fragment, antigen-binding fragment, single domain antibody, and single-chain variable fragments are used. Advantages of recombinant fragments include lower molecular weight and thus higher penetration ability,

Figure 1

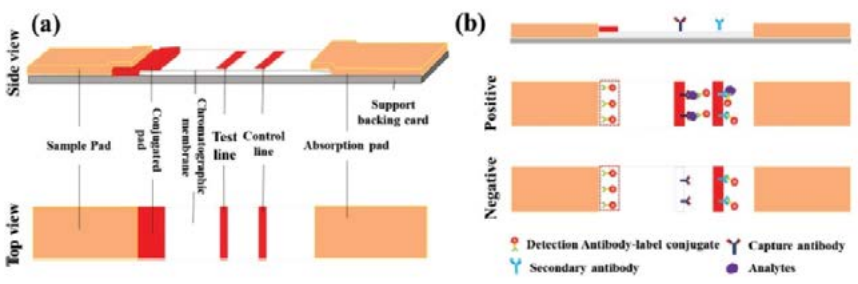


Fig. 1: *a) Typical configuration of an LFA. (b) Schematic diagram of sandwich LFA.*

high antigenic affinity, and mass production in multiple protein expression systems.

Sometimes nucleic acid aptamer, an oligonucleotide which was selected to specifically bind its target antigen is used. The aptamer folds into a well-defined 3D structure, and upon binding to its target antigen adopts a different configuration, which can be easily measured.

Capture biomolecules

The core reagents in the Test (T) zone and Control zone are called the capture biomolecules. They are immobilized on the nitrocellulose membrane. In the C zone, the capture biomolecule captures the detection biomolecules in the sample flow and it induces a positive signal, providing a confirmation of the validity of the test strip: i.e., all reagents are working. In the T line, the capture antibody binds the detection antibody complexed with target antigen. With the presence of signal molecules, the capture biomolecules transform a chemical reaction which produces a readable signal.
The two essential features of capture biomolecules are

1. Rapid binding kinetics to ensure it captures the detection molecule-target antigen complex as it flows past the T line.
2. No interference in binding the target antigen between the detection biomolecule binding site and the capture biomolecule binding site.

Many capture biomolecules are antibodies and receptors but may also be polyclonal antibodies.

Labeling materials

Multiple types of labeling nanoparticles such as magnetic nanoparticles, metal nanoparticles and Quantum Dots (QDs) are used in LFAs. A commonly used labeling material is gold nanoparticles (AuNPs).

Gold nanoparticles

Gold nanoparticles can be produced to match the desired particle size range and shapes (Figure 2). Different sized AuNPs exhibit an absorption peak at 510-550 nm wavelength. Most LFAs use AuNPs in the range of 15-100nm. The larger AuNPs particle size causes a darker colloidal solution. The absorption peak of larger particle sizes can shift to a longer wavelength. Aggregation of many AuNPs induce a significant color shift: this property underlies its use in colorimetric immunoassays.

AuNPs can be attached to detection biomolecules via conjugation (covalent binding) or physical absorption. The size of the AuNPs should be tested empirically to balance the stronger light absorption of larger particles and their greater aggregation with lowered solubility in the sample solution. Figure 3a shows the experimental protocol and 3b shows that the NP density as well as particle volume on the T line is proportional to the minimum measurable optical signal.

Silver NPs (AgNPs)

AgNPS have a high extinction coefficient and smaller size than AuNPs, but they are less stable, have limited biocompatibility and are more challenging to maintain function. In recent years, the synthesis, stabilization, and functionalization of AgNPs have

Figure 2

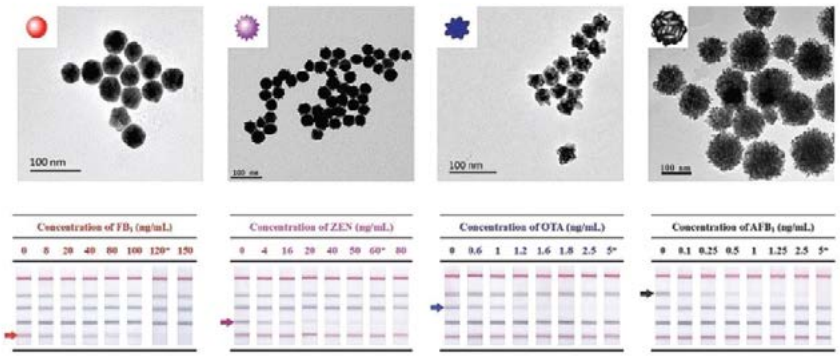


Fig. 2: *Gold nanoparticles as LFA labels: a) TEM images of four different shaped AuNPs, including gold nanospheres (AuNSs, red), gold nanocacti (AuNCs, purple), gold nanoflowers (AuNFs, blue), and hyperbranched Au plasmonic blackbodies (AuPBs, black), and their stereograms as LFA labels.*

Figure 3

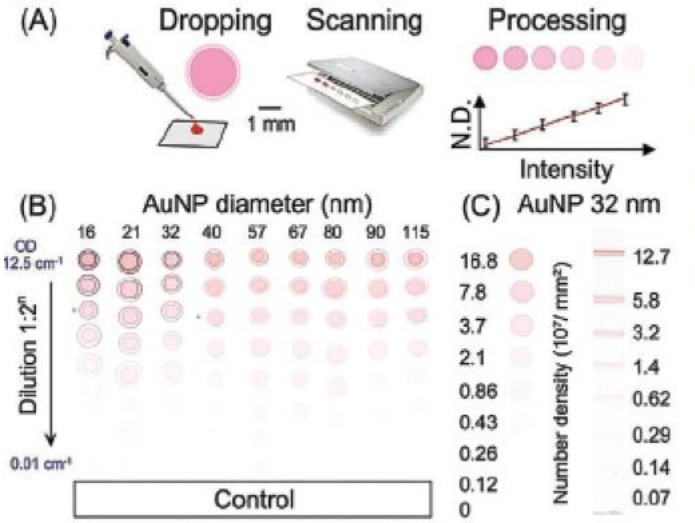


Fig. 3: *Gold nanoparticles as LFA labels for quantification of the AuNPs number in the spot. AuNPs, gold nanoparticles. Reproduced with permission. [Khlebtsov et al ACS Appl. Nano Mater. 2019, 2, 5020] Copyright 2019, American Chemical Society.*

Figure 4

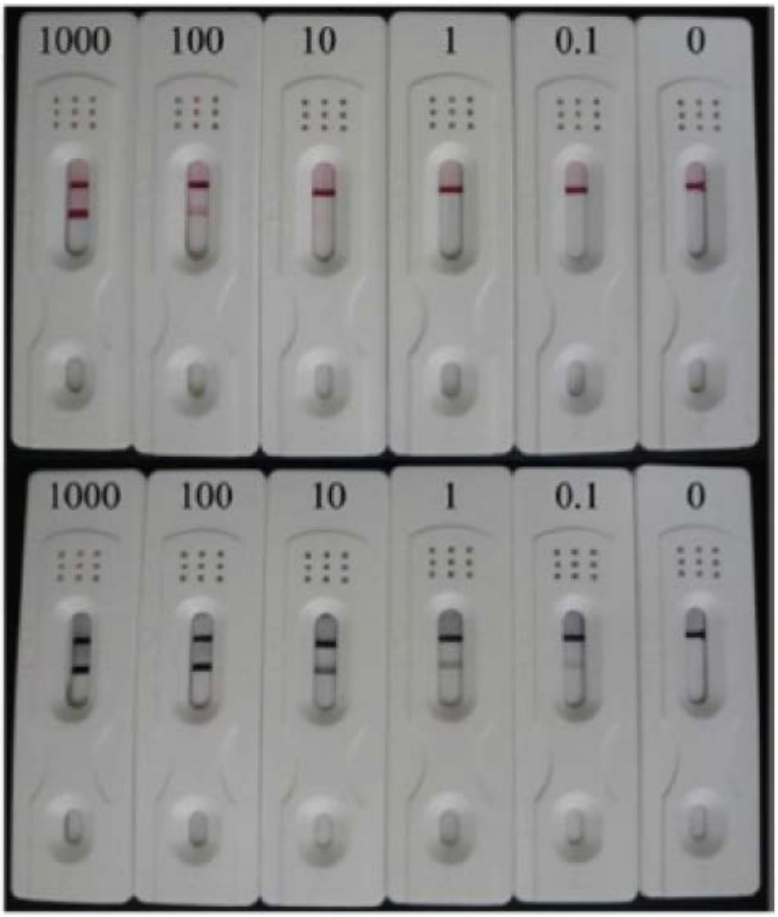


Fig. 4: *Silver enhancement of AuNP signal occurs in a dose dependent manner. The T line and C line in a signal enhanced LFA. https://www.sciencedirect.com/science/article/abs/pii/S0956566311001023 Reproduced with permission. Copyright 2011, Elsevier.*

Figure 5

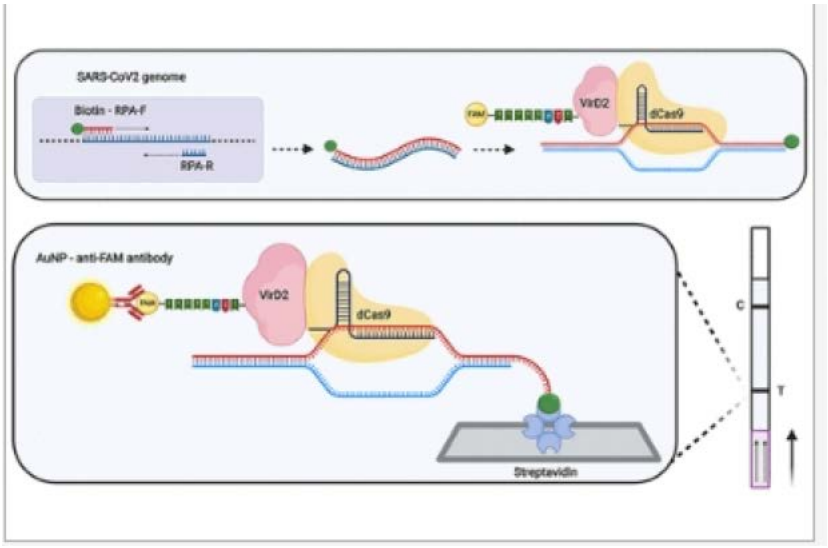


Fig. 5: *Schematic of Vigilant LFA to measure SARS-CoV-2 in clinical samples. It involves a VirD2-dCas9 guided and LFA-coupled nucleic acid test with reverse transcription-recombinase polymerase amplification. https://pubs.acs.org/doi/full/10.1021/acs.nanolett.1c00612 Abstract (open access.)*

improved, leading to greater usage in LFAs.

Silver can be used to enhance the signal from AuNPs in LFAs. This classic electroless silver enhancement process often uses silver lactate or silver acetate as source of silver ions, and the reducing agent is often hydroquinone buffered to an acidic pH. Briefly, AuNPs act as a catalyst and reduce silver ions to metallic silver, thereby enhancing the AuNP signal, as shown in the example in Figure 4.

Other labeling materials will be discussed in a later section.

LFAs for SARS-CoV-2

Five distinct strategies have been applied to development of an LFA for detection of SARS-CoV-2. First, Tan and coworkers [https://doi.org/10.1021/acs.analchem.0c05059] combined a one-pot direct reverse transcription-loop-mediated amplification (RT-LAMP) with a LFA format. The sample collection, treatment, isothermal amplification, and signal readout of the RT-LAMP-LFA system utilized 2, 5, 30, and 3 min, respectively. They claimed a >99% accuracy.

Mahfouz's group developed the Vigilant LFA assay which used a VirD2-Cas9 complex in their LFA for SARS-CoV-2 to reduce false positives due to primers and primer-dimers (Figure 5). [https://pubs.acs.org/doi/full/10.1021/acs.nanolett.1c00612] Briefly, the Vir2-Cas9 complex includes a catalytically inactive SpCas9 endonuclease fused to VirD2 relaxase to enhance specific nucleic acid detection. The Cas9 region specifically binds the target nucleic acid sequence and the Vir2 region covalently binds to a FAM-tagged oligonucleotide. The target nucleic acid is amplified and labeled with biotinylated oligonucleotides. The limit of detection is 2.5 copies/µL. It showed no cross reactivity to MERS or SARS-CoV-1.

Nichol's group [https://pubs.acs.org/doi/full/10.1021/acs.analchem.0c01975] used antibody specific for N protein to develop a half-strip LFA for detection of SARS-CoV-2. As previously mentioned, choice of the antibody for high affinity and other favorable attributes are the most important decision for development of the LFA. The limit of detection for the described LFA targeting a recombinant N protein is 0.65 ng/mL (95% CI: 0.53 – 0.77ng/mL).

Detection of antibodies to one or more of the SARS-CoV-2 antigens provides a quick and specific strategy to develop a Point-of-Care test. Zeng et al reported on a LFA based on detection of IgG and IgM antibodies against SARS-CoV-2 antigens or its recombinant antigens in clinical blood samples

Figure 6

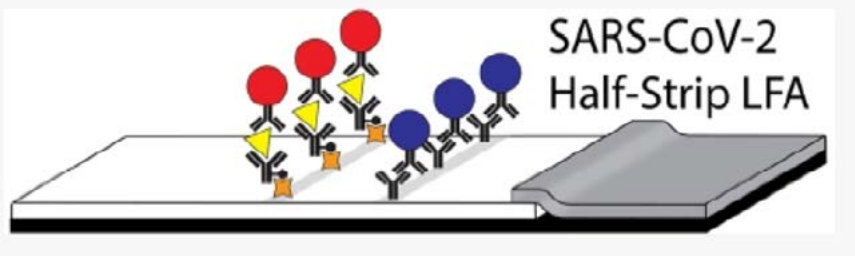


Fig. 6: *N protein based half-strip LFA for detection of antibodies specific for SARS-CoV-2. The Control line binds the detection antibody (blue circle)*

Figure 7

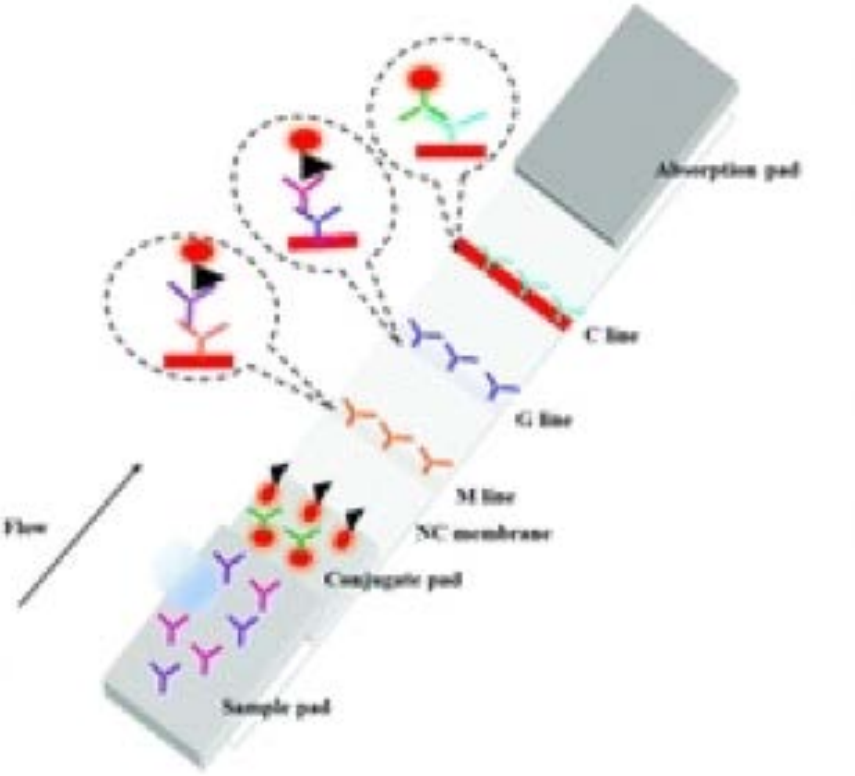


Fig. 7: *Sandwich LFA test that can detect IgM- and IgG-specific for antigen(s) of SARS-CoV-2. [https://pubs.rsc.org/en/content/articlelanding/2020/qm/d0qm00294a/unauth]*

Figure 8

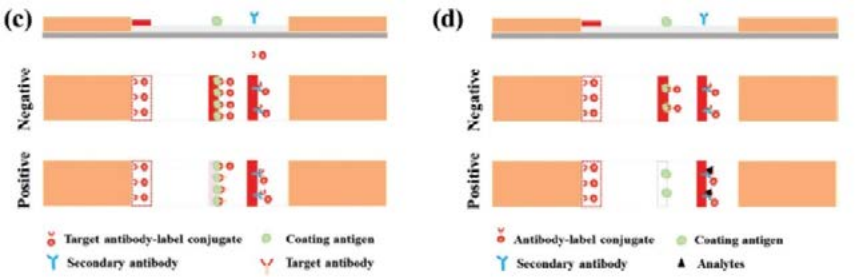


Fig. 8: *Common structure of an indirect competitive LFA (c) and a direct competitive LFA (d).*

within 15 min (Figure 7). [https://pubs.rsc.org/en/content/articlelanding/2020/qm/d0qm00294a/unauth] The LFA provided two Test lines, one for detecting IgG- and one for detecting IgM-specific for SARS-CoV-2 antigen. The SARS-CoV-2 recombinant antigen was conjugated to AuNPs, and generated a red line in samples containing the relevant IgM or IgG antibodies. In hospitalized COVID-19 patients, the sensitivity of the assay was 85.3% and specificity was 100%. These results indicate that an LFA detecting both IgG- and IgM-specific SARS-CoV-2 antibodies had higher sensitivity than LFAs that measured only one type of antibody (IgG or IgM).

Detection of neutralizing antibodies to SARS-CoV-2 by LFAs

Neutralizing antibodies (NAbs) to SARS-CoV-2 target the S protein, which has a region called the receptor- binding domain (RBD) that binds the ACE2 receptor on numerous cell types. Basically, NAbs and the receptor ACE2 compete for binding to the S protein. Two types of competitive LFA formats, indirect competitive LFA and the direct competitive LFA have been developed for other targets and are depicted in Figure 8.

The authors concluded that LFA formats offer a broad repertoire for development of suitable LFAs. Furthermore, changes are being pursued to improve their sensitivity and accuracy as well as their stability during storage and standardized software for smartphone readouts.

BACK TO CONTENTS

Alkaline Phosphatase-based Electrochemical Analysis for Point-of-Care Testing

Kanno Y, Zhou Y, Fukuma T *et al.*

Article

Many laboratory tests and point-of-care (POC) devices use enzymes to facilitate highly selective detection of many biological molecules, biomarkers, and ions. Enzymes such as alkaline phosphatase (ALP) are commonly used to generate and amplify detection signals of recognition components, such as antibodies or receptors. This review describes research advances in ALP-based approaches for POC testing on two main topics: ALP substrates for electrochemical signaling and ALP-based devices for POC testing.

The main ALP substrates are listed below:

- *p*-Aminophenyl phosphate (PAPP)
- *p*-Naphthylphosphate (1-NPP)
- L-Ascorbic acid phosphate (AAP)
- *p*-Nitrophenyl phosphate (PNPP)
- Ferrocenylethyl phosphate ($FcEtOHPO_3$)$^{-2}$

Characteristics for the ideal substrate for POC tests include chemical stability, low cost, free of electrode fouling, highly electrochemically reactive, and commercially available.

p-Aminophenyl phosphate (PAPP)

ALP can remove the phosphate monoesters from the substrate PAPP and convert it to PAP. The electroactive p-aminophenol (PAP) can be oxidized to p-quinoneimine (PQI) in the presence of several electrodes such as Au, Platinum (Pt), and carbon-based electrodes. The PAP/PQI electrochemical reactions are reversible, and redox cycling between the cathode and anode can enhance the signal-to-background ratio.

Devices that include recognition components for biomolecules have been developed for POC testing. ALP's very suitable substrate PAPP can be further enhanced with signal amplification strategies, such as redox cycling. The oxidation potentials of PAPP and PAP do not overlap, and each can be separately detected.

Development of PAP/PQI redox cycling-based chips have included nanocavity electrodes, interdigitated array electrodes, and vertically separated electrodes, as depicted in Figure 1.

However, in alkaline solutions PAP is polymerized to form a brown deposit, which can alter the signal. Multiple groups have decreased the pH of the PAPP solutions to avoid this complication.

1-Naphthylphosphate (1-NPP)

ALP reactions can transform 1-NPP to an electroactive species 1-naththol (1-NP). Because the oxidation potentials of 1-NPP and 1-NP do not overlap, the electrochemical signals of 1-NP can be measured in the presence of 1-NAP with either Au and carbon-based electrodes. Note that 1-NP can be converted irreversibly to an electropolymerized derivative and polymerization leads to signal decay and fouling of electrodes.

L-AAP

ALP reactions can convert AAP to an electroactive species, L-ascorbic acid (AA). Several ALP-based electrochemical bioassays have used AA as a reducing agent [https://pubs.acs.org/doi/10.1021/ac5028885]. The suitability for ALP-based electrochemical immunoassays that used glassy carbon electrode, Au electrode, and screen-printed carbon electrode was examined for the following 7 electroactive species: AA, indigo carmine, hydroquinone, 1-NP, PAP, and phenol [https://doi.org/10.1016/j.ta-

Figure 1

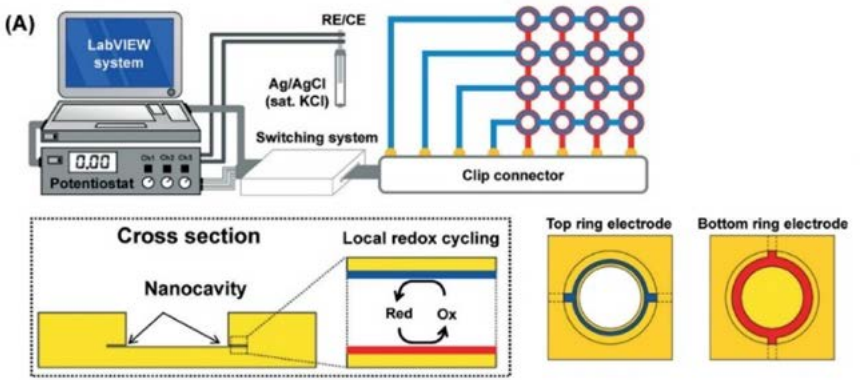


Fig. 1: *Schematic illustration of ALP detection using PAPP and redox cycling. The local redox cycling-based device has nanocavities and microwells. It contains 4 rows of the top ring electrodes (blue) and 4 column bottom ring electrodes (red). The ring electrodes are placed at individual crossing points and separated by nanocavities. The microwell arrays are made at the individual crossing points where redox cycling are locally induced.*

Table 1

Sensor type	ALP substrate	Target	Working electrode material	Ion Dependence	References
Immunosensor	PAPP	Malarial histidine-rich protein	Carbon	NA	https://pubs.acs.org/doi/10.1021/ac5020782
	1-NPP	Urinary metabolite	Carbon	NA	https://pubs.acs.org/doi/10.1021/acsami.9b16193
	AAP	Endocrine hormones	MoS_2-Au	NA	https://pubs.acs.org/doi/10.1021/acs.analchem.9b05172
Aptasensor	1-NPP	Allergenic protein	Carbon	NA	https://doi.org/10.1002/elan.201900318
	1-NPP	Mycotoxin	Carbon	NA	https://doi.org/10.1016/j.bioelechem.2020.107691
	1-NPP	Secretory glycoprotein	Carbon	NA	https://doi: 10.1016/j.talanta.2019.120666
DNAzyme sensor	1-NPP	Pb^{2+}		NA	https://doi.org/10.1016/j.bios.2016.04.026
	1-NPP	DNA		Mg^{2+}	https://doi.org/10.1016/j.bios.2010.04.012
	PAPP	DNA		Zn^{2+}	https://doi.org/10.1016/j.bios.2014.07.078

Mg^{2+}, magnesium ion; NA, not applicable; Pb^{2+}, lead ion; Zn^{2+}, zinc ion.

Table 1: *Comparison of ALP-based electrochemical devices for POC testing*

lanta.2008.03.025]. AA is both affordable and nonfouling for the aforementioned types of electrodes and was chosen for its potential in reusing POC devices. Note that AA can foul Pt electrodes, and also graphic electrodes at high concentrations (≥50μM).

p-Nitrophenyl phosphate (PNPP)

ALP reactions can convert PNPP to a yellow PNP, which can be measured in colorimetric assays. Although PNPP is as inexpensive a reagent as AAP, the chemistry of PNP is more complex, often inducing 1-NP. Note that the oligomerization products of 1-NP often fouls electrodes and after each measurement, a polishing process is needed to maintain performance.

Ferrocenylethyl phosphate ($FcEtOPO_3^{2-}$)

Ferrocenylethyl phosphate is not commercially available and thus, its application in development of ALP-based POC test is more challenging.

ALP-based analytical devices

Three types of ALP-based electrochemical devices are discussed in this review: immunosensors, aptasensors, and DNAzyme sensors (Table 1).

ALP-based immunoassays are common (e.g., enzyme linked immunoassay, ELISA). They usually link the ALP enzyme to the recognition biomolecule (eg, antibody). The immunological reagents are immobilized on a specialized plastic plate. Blocking with a specific protein solution (eg, casein) is often used to reduce nonspecific signals.

As an alternative format, paper-based devices used a central crease to separate two zones of hydrophobic cellulose paper (Figure 2). The two zones comprised an

Figure 2

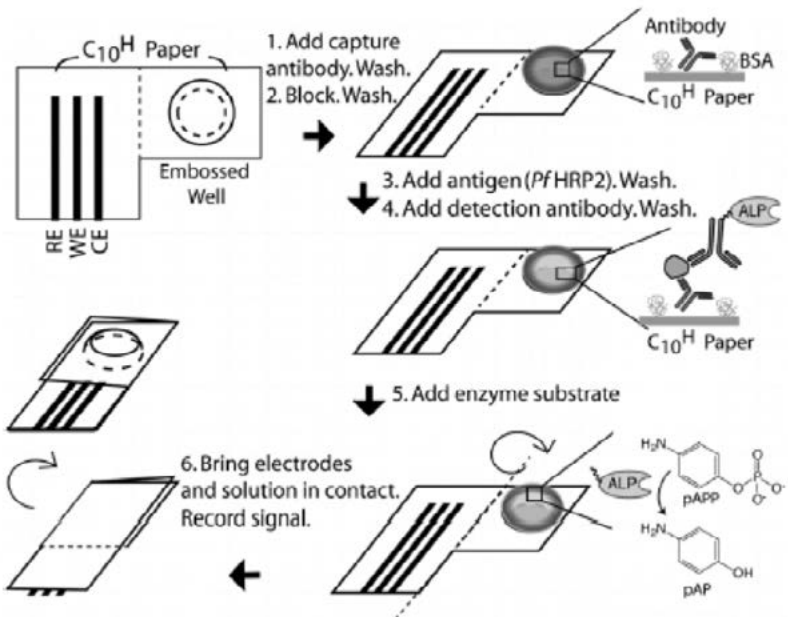


Fig. 2: *Schematic of the 6 steps involved in the paper-based electrochemical immunosensors. The six steps are (1) immobilization of capture antibodies, (2) blocking nonspecific binding, (3) binding of target antigen to specific antibody, (4) binding of ALP-linked antibody (detection antibody) to target antigen, (5) PAPP substrate added to microwell and ALP converts PAPP to PAP in dose dependent manner, (6) measurement of electrochemical signal. Reprinted with permission. [https://pubs.acs.org/doi/10.1021/ac5020782]*

embossed microwell for recognition of the analyte and a detection zone.

Recognition components of immunoassays now include nanobodies which are composed of a single variable domain of the heavy chain of camelid antibody (no light chains). Compared to traditional antibodies, advantages of nanobodies include ease of cloning and expression, high solubility, thermal stability, and chemical stability. An ALK-based POC testing device can be used to measure metabolites from pyrethroid pesticides [https://pubs.acs.org/doi/10.1021/acsami.9b16193]. They used a different membrane (chemically modified hydrophilic nylon nanofibrous membranes) and fabricated a microporous area for improved target access. The authors expect that nanobodies will be used in more POC platforms.

Figure 3

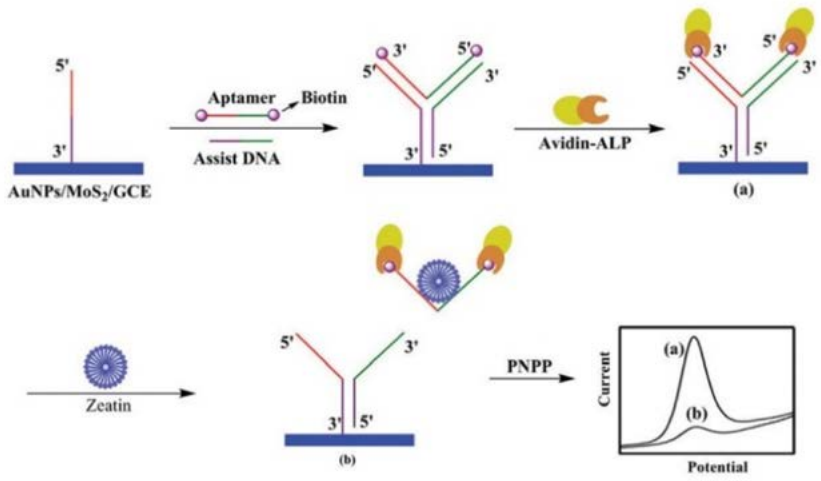


Fig. 3: *Schematic illustration of an electrochemical aptasensor for detecting zeatin. The Y-type DNA-based aptasensor was manufactured with an AuNP electrode with molybdenum disulfide (MoS_2) nanosheets. The aptamer DNA was modified with biotin at both terminals. Zeatin, when in sample, binds to aptamer and collapses the Y-type DNA nanostructure, which disrupts the ALP oxidation of PNP and reduces the electrochemical signal. Reproduced with permission. [https://pubs.rsc.org/en/content/articlelanding/2018/AN/C8AN01356J]*

Figure 4

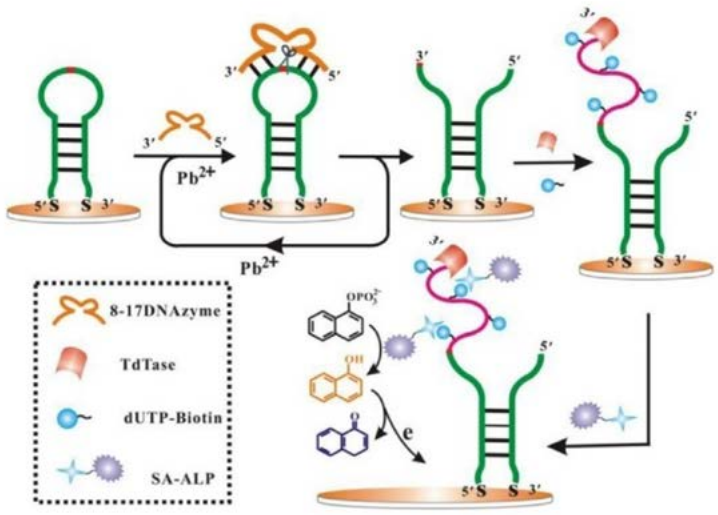


Fig. 4: *Schematic illustration of DNAzyme-based electrochemical sensor for Pb^{2+}. The biosensor uses the DNAzyme, a terminal deoxynucleotide transferase (TdT) -mediated extension, and streptavidin-labeled ALP produced electrochemical signal. https://doi.org/10.1016/j.bios.2016.04.026*

Aptasensors

The single-stranded oligonucleotides called aptamers can be designed to bind to bacterial and viral analytes with high specificity and affinity, rivaling the antigen-antibody interactions. Their advantages over traditional antibodies include ease of chemical modification, high thermal stability, and high productivity.

Aptasensors which are electrochemical aptamer-based sensors involve immobilization of the thiolated aptamers on Au nanoparticles or carbon electrodes. The detection of the analytes is often based on analyte binding-induced changes in the folded DNA nanostructures (eg, arch, ladder, and M types), as illustrated in Figure 3.

Thus, the aptamer-analyte complex changes the DNA nanostructure and allows measurement of the analyte.

DNAzyme sensors

The single-stranded DNA molecules called DNAzymes exhibit catalytic functions, like enzymes. Binding of the DNA sequences to specific target sequences alters the catalytic functions. Two advantages of DNAzymes for POC testing are their stable catalytic functions under extreme pH conditions and high temperatures. DNAzymes include peroxidase-mimicking DNAzymes and metal-ion dependent RNA cleaving DNAzymes.

A DNAzyme-based sensor to measure Pb^{2+} is shown in Figure 4 [https://doi.org/10.1016/j.bios.2016.04.026]. After immobilization of the hairpin-like substrate strand (HP DNA) onto the electrode, a sample containing Pb^{2+} allows the 8-17 DNAzyme to cleave the HP DNA and expose the 3'-OH for extension by terminal deoxynucleotide transferase (TdT) which incorporates biotinylated dUTP. The streptavidin-labeled ALP converted the electrochemical substrate 1-NPP to the electrochemically active species 1-napthyl phosphate.

The DNAzyme-based sensor used two amplification strategies to obtain a detection limit of 0.43 nM Pb^{2+}. It offers a prototype for development of DNAzyme-based POC tests for detection of metal ions and other nucleic acid-related analytes.

An HRP–labeled Lateral Flow Immunoassay for Rapid Simultaneous Detection and Differentiation of Influenza A and B viruses

Zhang J, Gui X, Zheng Q *et al.*

Article

Epidemics can also be caused by Influenza (INF) viruses, inducing significant morbidity and mortality. Since early detection and control would improve clinical decision making, rapid diagnostic tests are warranted. The three current methods used in laboratory settings are time consuming and require specially trained personnel and expensive equipment. Thus, additional methods beyond immunofluorescence assay, virus isolation via cell culture, and real-time PCR are needed, especially in resource-poor areas.

Lateral flow immunoassays (LFA) can be rapid and simple, and are often used in non-laboratory or resource-poor environments. Sensitivity and limit of detection are the main challenges for LFA that use current labels such as gold nanoparticles, fluorescent particles, up-converting phosphors, and quantum dots. Zhang et al describe a new platform for LFA for detection of INF A and INF B with the following advantages: enhanced sensitivity, few steps, and a visible signal readable by eye. The well-established enzymatic activator, horseradish peroxidase, is stable, cost effective, and easy to prepare.

Figure 1

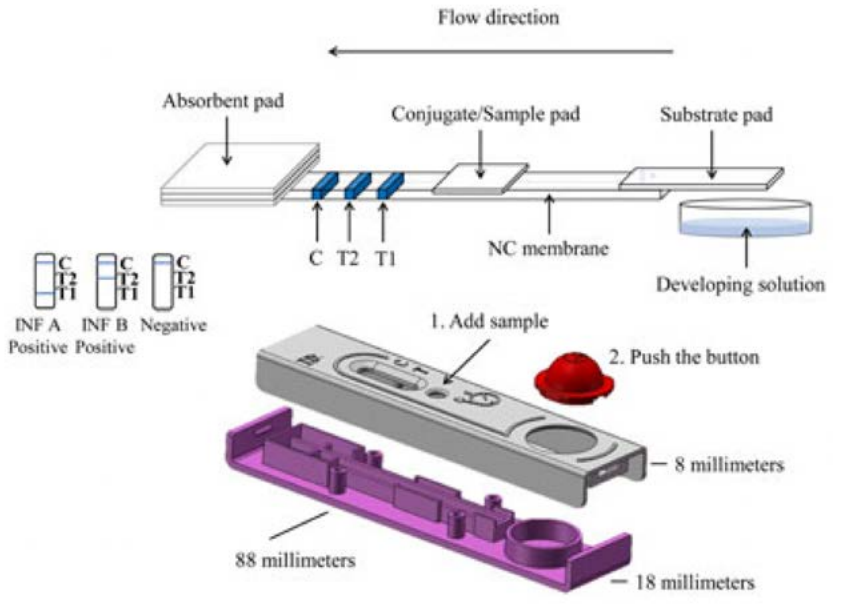


Fig. 1: *The principle of the HRP-LFA and its procedure for detection of INF A and INF B viruses. C, control line (no virus) GAM-IgG antibody; T2, test line 2, monoclonal antibody 1B9 reactive against INF B NP protein; T1, test line 1, monoclonal antibody 19C10 reactive against INF A NP protein; NC, nitrocellulose membrane.*

Development of HRP-LFA for detection of NP to INF A and B.

Figure 1 illustrates the concept and construction of the all-in-one HRP-LFA device configured in a strip. The main components include:

- Conventional backed nitrocellulose membrane (Hi–Flow Plus 135; Millipore Corp (Billerica, MA))
- Conjugate/sample glass fiber pad (soaked in antibody–HRP conjugate dissolved in buffer solution)
- Absorbent pad
- Substrate pad (At appropriate time, with addition of 5 microlitres of 0.1 mmol/L 3,3′,5,5′–tetramethylbenzidine (TMB, Sigma))
- Developing solution [0.5mmol/L urea peroxide and 0.01 mmol/L dextran sulfate in citrate buffer adjusted to a pH of 5.0
- Goat anti-mouse (GAM)-IgG antibody (DAKO Corporation or Wantai, Beijing)
- Casein sodium, HRP, and polyethylene glycol (MW20000) from Sigma
- CH37K filter paper, polyvinylchloride backing sheet, SB06 glass fiber from KinbioTech Co. LtD (Shanghai, China)
- Four monoclonal antibodies (19C10, 11F12, 1B9, 10B6) that bind cluster epitopes in NP for INF A and INF B were selected from immunized BALB/c mice. HRP-11F12 and HRP-10B6 conjugates at a mass ratio of 1:1 were stabilized in 50% glycerol and stored at -20°C

For setup, a matrix reagent dispensing module was used to spray the capture antibodies (2 mg/mL) and GAM-IgG antibody on the XYZ3050 Platform (BioDot, Irvine, CA). The 5 mm cut strips (ZQ2000 guillotine cutter, Kinbio Tech) of the assembled membrane were stored at room temperature in vacuum-sealed bags.

Procedure

The entire procedure can be completed in 20 minutes. After the 50 µL of liquid specimen obtained from oropharyngeal swab or nasopharyngeal swab was pipetted onto the sample pad, depression of the red button soaks the substrate pad in the developing solution. The control C line should develop a blue colorimetric signal. When INFA or INFB or both viruses are present at sufficient titers, the T line(s) also develop a blue colorimetric signal. The results (colorimetric signals) can be read visually in 20 minutes.

In principle, when the positive sample is loaded onto the conjugate/sample pad, the NP of the sample forms an immune complex with the anti-NP antibody-HRP in the pad. The developing solution in the now wet substrate pad induces the immune complex to diffuse toward the detection zone by capillary force. At the detection zone, the immune complex reacts with the specific anti-NP antibodies, and the HRP in the complex induces a dark blue color by oxidizing the TMB substrate.

Analytical sensitivity of HRP-LFA compared to colloidal gold-based LFA.

The commercially available BD Directigen EZ Flu A+B uses colloidal gold strips in its LFA. Its limit of detection (LOD) for INF A samples ranged from 0.01 to 0.04 HA titer and for INF B, from 0.02 to 0.32 HA titer. Analytic sensitivity of the HRP-LFA and the BD Directigen EZ Flu A+B were compared in eight INF A isolates and five INF B isolates (Table 1). The LOD for INF A with the HRP-LFA ranged from 0.00025 to 0.0001 HA titer, which is at least 10x more sensitive than the colloidal gold-based LFA (BD). Similarly, the LOD for INF A with the HRP-LFA ranged from 0.016 to 0.004 HA titer, which is at least 5x more sensitive than the colloidal gold-based LFA (BD Directigen).

Experiments were performed in triplicate. HRP, horseradish peroxidase; LFA, Lateral flow immunoassay; HA titer, reciprocal of viral dilution with complete hemagglutination; LOD, limits of detection.

Cross reactivity to common pathogens of the respiratory tract were evaluated with the HRP-LFA (Table 2). Cell culture infected with one of these 5 pathogens (Adenovirus, Enterovirus, Mycoplasma pneumoniae, Newcastle disease virus, and Respiratory Syncytial Virus) was diluted in PBS and tested. No cross reactivity was detected (Table 2). The INF A and INF B control samples all tested positive (n=10).

Table 1

Virus Strains	LOD (HA titer) HRP-LFIA	BD Flu A+B
A/California/04/2009 (H1)	0.0005	0.01
A/Xiamen/N66/2010 (H1)	0.001	0.02
A/Xiamen/042/2009 (H3)	0.0005	0.01
A/Xiamen/067/2007 (H3)	0.001	0.04
A/Hong Kong/YU22/02 (H5)	0.00025	0.01
A/Fujian/897/2005 (H5)	0.0005	0.01
A/Shantou/4253/2003 (H11)	0.001	0.02
A/Shantou/834/2001 (H11)	0.001	0.02
B/Xiamen/627/2007 (Yamagata)	0.004	0.04
B/Xiamen/756/2007 (Victoria)	0.016	0.32
B/Xiamen/1346/2008 (Victoria)	0.004	0.04
B/Xiamen/742/2008 (Yamagata)	0.004	0.02
B/Xiamen/013/2008 (Yamagata)	0.004	0.04

Table 1: *Analytical sensitivity of INF A&B HRP-LFIA for detecting virus isolates*

Sensitivity and Specificity

After approval by the Chinese CDC, 1487 swab specimens were collected from patients with influenza-like symptoms. The 773 oropharyngeal samples and the 714 nasopharyngeal swab samples were diluted in PBS and stored at -80°C at the Chinese CDC.

Quantitative PCR was used as the standard to test the specimens for the INF A and/ or INF B NP proteins. Briefly viral RNA was isolated and extracted from 200 µL specimens with commercially available kits. Reverse transcription and real-time PCR (RT-PCR) were performed by using commercially available kits: AccessQuick™ RT-PCR kit (Promega, Madison, WI) on a CFX96 Touch™ Real–Time PCR Detection System (Bio-Rad, Hercules, CA). The primers and probe for INF A and INF B viruses had been well-established.

Clinical evaluation indicated that nasopharyngeal swab samples had a 90.2% sensitivity for INF A but a lower sensitivity with oropharyngeal swabs (66.2%) (Table 3). Similarly, for INF B, nasopharyngeal swab samples had a 82.6% sensitivity for INF B but a lower sensitivity with oropharyngeal swabs (58.4%) (Table 3). These data suggest that the sensitivity of this HRP-LFA for INF A/B was higher in nasopharyngeal swab samples and that nasopharyngeal swabs should be considered to be a preferred sampling method. The specificity of these HRP-LFA INF A/B tests were very high, ≥99%.

Table 2

Pathogen	Test dosage	No. tested	No. positive
Adenovirus	TCID50/mL $10^{6.0}$	1	0
Enterovirus	TCID50/mL $10^{5.5\text{-}7.0}$	21	0
Mycoplasma pneumoniae	≥107 CCU/mL	1	0
New Castle Disease virus	TCID50/mL $10^{6.0}$	2	0
Respiratory Syncytial Virus	TCID50/mL $10^{6.0}$	2	0

Table 2: *Evaluation of cross reactivity of INF A/B HRP-LFA*

Table 3

	Influenza A		Influenza B	
Specimen type	**Sensitivity (%) (95% CI)**	**Specificity (%) (95% CI)**	**Sensitivity (%) (95% CI)**	**Specificity (%) (95% CI)**
Nasopharyngeal swab (n=714)	90.2% (80.2%–95.4%)	99.7% (98.7%–99.9%)	82.6% (73.2–89.1)	100.0 (98.7–100.0)
Oropharyngeal swab (n=773)	66.2% (54.3%–76.3%)	100.0% (99.3%–100.0%)	58.4% (47.3%–68.8%)	99.7% (98.8%–99.9%)
Total	77.5% (69.6%–83.9%)	99.8% (99.4%–100.0%)	71.2% (63.8%–77.6%)	99.8% (99.2%–99.9%)

CI, confidence interval; HRP, horseradish peroxidase; LFA lateral flow immunoassay.

Table 3: *Clinical evaluation of the HRP-LFA in comparison to RT-PCR*

Two modifications of previous HRP-LFA tests contribute to the higher sensitivity here: First, Zhang et al screened nitrocellulose membranes for a pore size that allowed efficient diffusion of the immune complexes. Second, the membrane was blocked with 1% casein to reduce nonspecific reactions and improve signal to noise ratio. In conclusion, these modifications increased the sensitivity of the HRP-LFA for INF A/B. This assay can serve as a prototype for development of HRP-LFA against other infectious agents.

Recent Advances and Clinical Application in Point–of–Care Testing of SARS–CoV–2

Ye Q, Lu D, Zhang T *et al.*

Article

Ye et al summarize the point-of-care (POC) testing developed for SARS-CoV-2 detection from three aspects: extraction of nucleic acids, nucleic acid amplification, and methods of detection. This review also describes the advantages and disadvantages of various instant detection methods and quality assurance. Nucleic acid POCT technology combines nucleic acid extraction, amplification, automatic detection with analysis of results.

Ye et al also present a commercially available real-time detection system that integrates nucleic acid extraction, amplification, and automatic detection. They also note potential issues during use.

Nucleic acid extraction

Five simplified nucleic acid extraction methods that can rapidly harvest the RNA from SARS-CoV-2 samples include high temperature lysis, solution lysis, solution lysis combined with high temperature, magnetic bead extraction method, and spin column-based method. They are illustrated in Figure 1. The first three extraction methods use a single step which does not require opening the lid at multiple steps in the extraction process, shortens the detection period, and supports simple operation in low resource environments. However, some

Figure 1

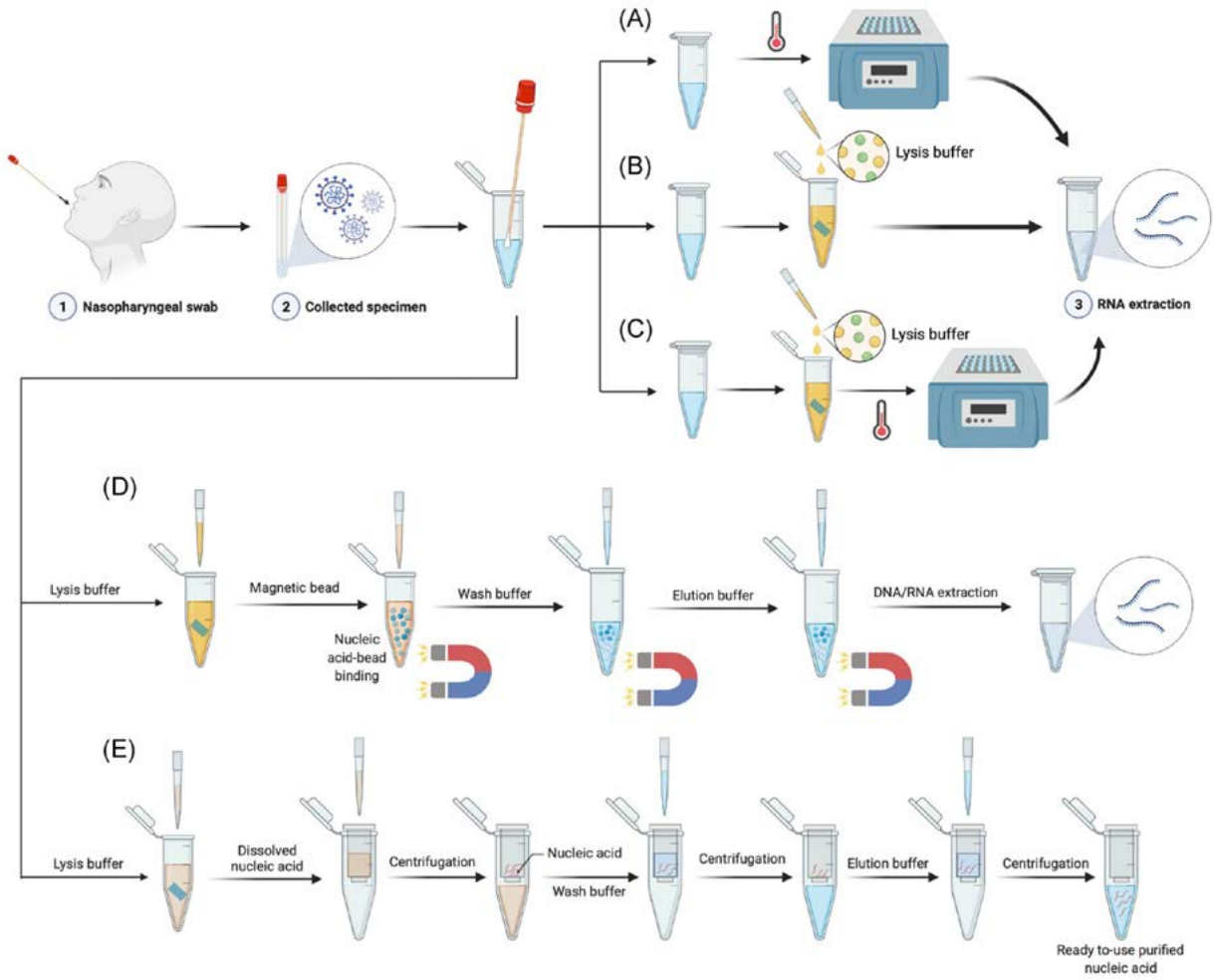


Fig. 1: *Schematic illustration of nucleic acid extraction methods for POC testing. Three simple extraction methods: (A) high temperature lysis, (B) solution lysis, (C) solution lysis combined with high temperature, and two multiple step nucleic acid extraction methods: (D) magnetic bead extraction method, and (E) spin column-based method.*

groups may prefer the multiple step magnetic bead extraction method or the spin column-based method extraction process.

DNA amplification

Four DNA amplification methods are being considered for POC testing.

Reverse transcription Polymerase chain reaction (RT-PCR)

RT-PCR is considered the gold standard for detection of SARS-CoV-2 genome and are supported by numerous commercially available kits (Figure 2A). The main challenges to incorporating RT-PCR into POC testing include the need for a thermal cycler (relatively expensive equipment), professional staff for operation, bulky and complex real-time fluorescence detectors, and extended turnaround time.

Isothermal amplification of nucleic acid

Isothermal amplification does not require a thermal cycling step and can detect viral RNA at similar levels to RT-PCR. The most common isothermal amplification technologies being investigated for real-time detection include loop–mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), and nicking enzyme–assisted reaction (NEAR), as illustrated in Figure 2.

Loop–mediated isothermal amplification (RT-LAMP)

RT-LAMP uses 3 basic steps:

- reverse transcription of RNA genome of SARS-CoV-2
- addition of 4 specially designed primers that bind 6 regions of SARS-CoV-2 genome
- generation of single-stranded template by DNA polymerase with strand displacement activity

The reaction is run at 60°C-65°C and can be completed in less than an hour. A positive result can be visualized by change from red to yellow and limit of detection is 118.6 copies /25µL. The reaction has high sensitivity, reproducibility, and specificity. Because of the need for a water bath or hot plate which limits its portability and miniaturization, RT-LAMP may not be the ideal nucleic acid amplification method. Methods run at ambient temperature would be more suitable.

Recombinase polymerase amplification (RPA)

RPA can be performed at normal temperature and depends on three enzymes:

- Recombinase which binds single-stranded nucleic acids

Figure 2

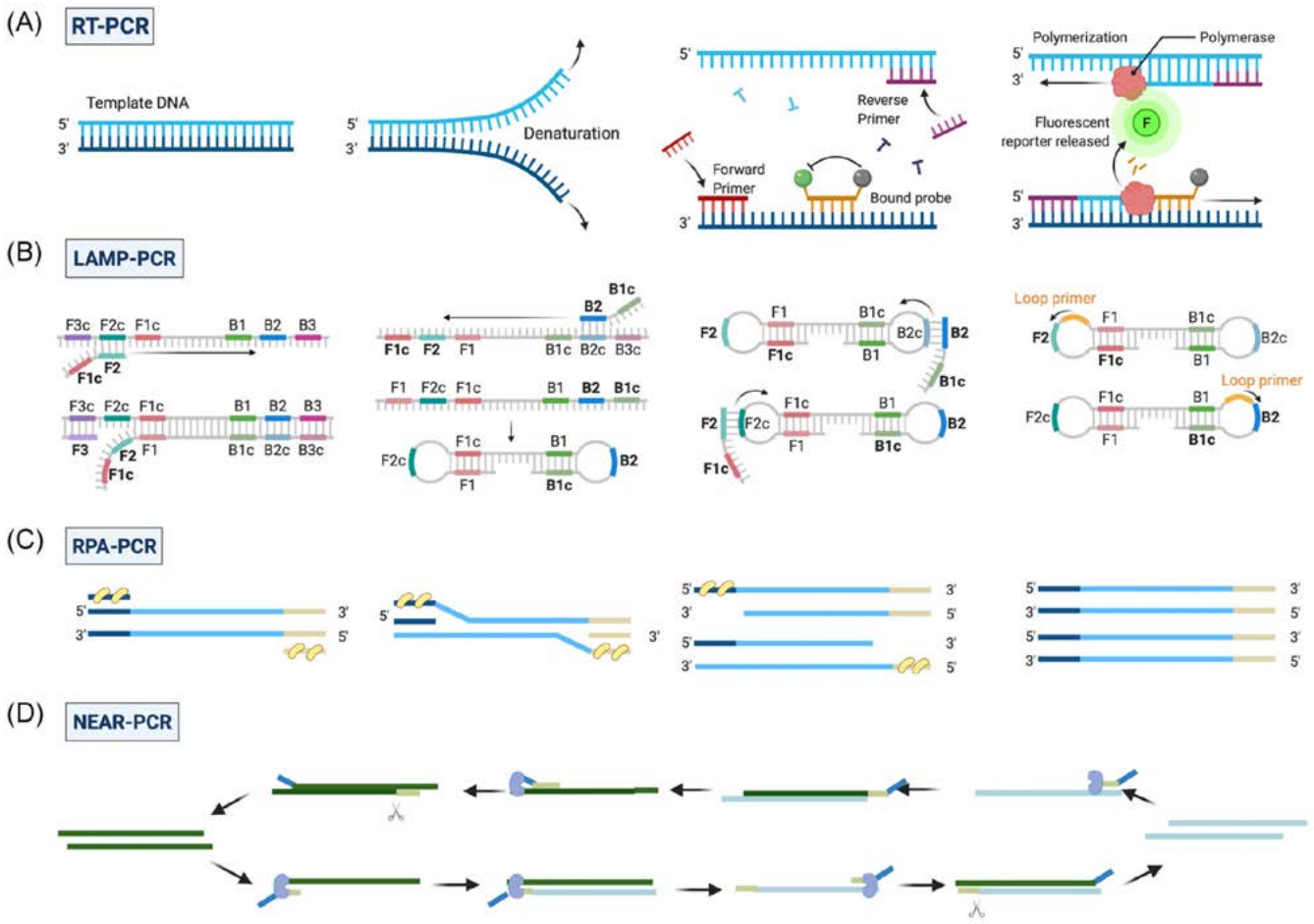


Fig. 2: *Common isothermal amplification technologies being investigated for real-time detection. (A) reverse transcription polymerase chain reaction (RT-PCR) (B) loop–mediated isothermal amplification (LAMP), (C) recombinase polymerase amplification (RPA), and (D) nicking enzyme–assisted reaction (NEAR)*

- Single-stranded DNA binding protein (SSB)
- Strand displacement DNA polymerase

The recombinant enzymes and primers for a protein DNA complex which targets the relevant sequences in SARS-CoV-2 template DNA. After strand displacement, DNA synthesis is initiated and the target region is exponentially amplified. Real-time fluorescence quantitation can be combined with an info probe to use lateral flow strips and visually detect or read results (with dye). The single tube RT-RPA method can obtain results in <10 min. and is consistent with RT-qPCR results. The limit of detection is reported as < 8 copies per reaction. However, the method is protected by patent which may limit its wide spread development.

Nicking enzyme–assisted reaction (NEAR)

NEAR uses the three types of enzymes: reverse transcriptase, nicking enzymes, and isothermal amplification DNA polymerase. The primer hybridizes with the template and its extension product is replaced and extended to form the complementary nicking enzyme recognition site. The target

Figure 3

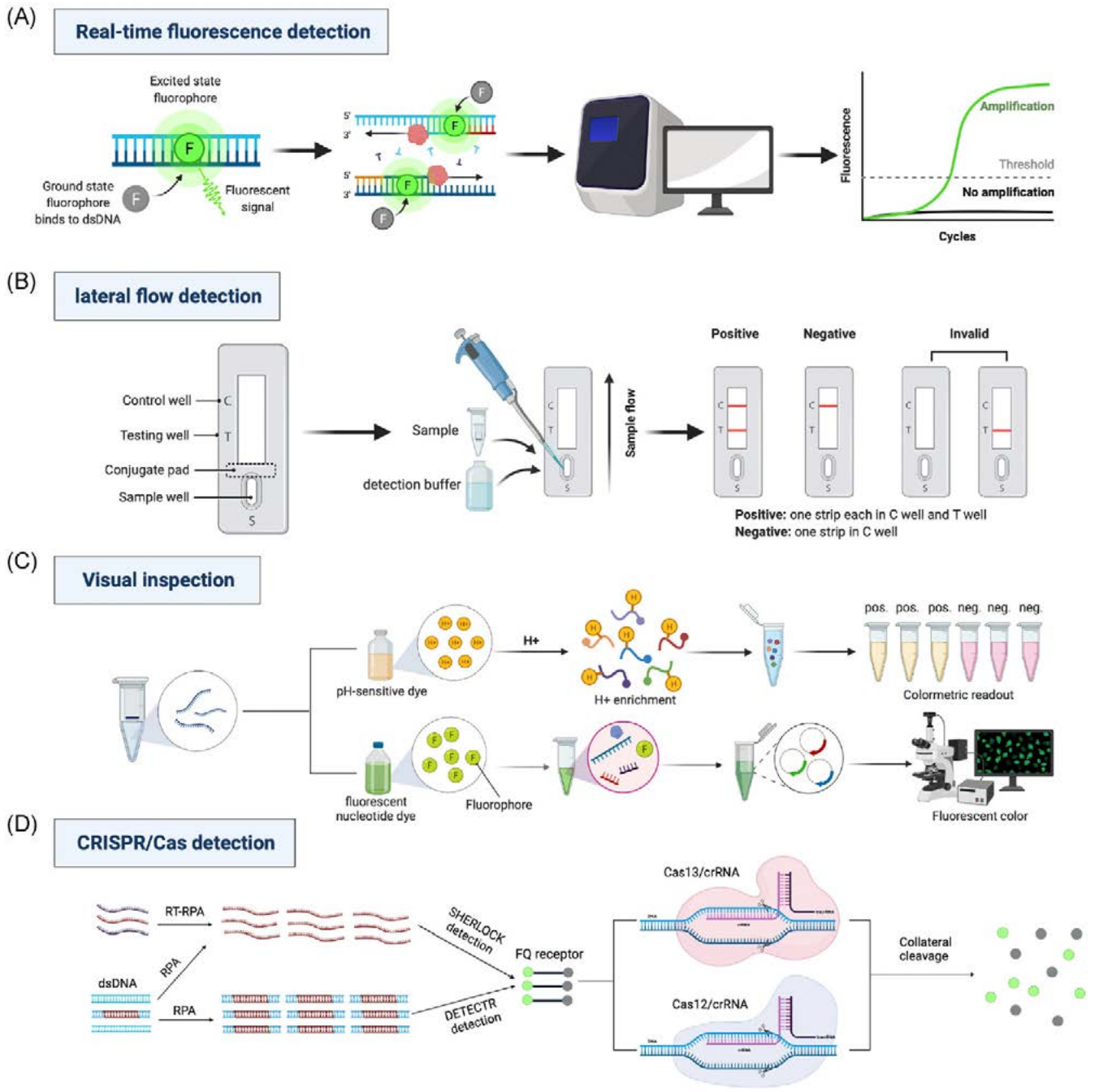


Fig. 3: *Detection method in point–of–care testing of SARS–CoV–2. Schematic diagram of Real–time fluorescence detection (A), Lateral flow detection (B), Visual inspection (C), and CRISPR/Cas detection (D)*

DNA template is exponentially amplified and labeled with fluorescent signals for quantitation. NEAR has a high sensitivity and reaction speed but the design of short sequences can increase the rate of false positives.

Detection Methods

The detection methods are illustrated in Figure 3.

Real-time fluorescence detection

Chemiluminescent substances can be added to the system to amplify the signals and increase quantification. The RT-PCR method can use fluorescent dyes such as SyberGreen. In comparison, the TaqMan probe method involves a fluorophore labeled 5′ primer and a 3′ quenching group to hybridize with the DNA template. Taq polymerase cleaves the fluorescent group which then fluorescence. Quantification usually is determined by the number of cycles at the fluorescent threshold which is performed by a specialized real-time reader. The need for specialized instruments appears to limit its popularization and broad application in POC testing.

Lateral flow detection

Paper-based LFA are low cost, easy to manufacture, and are used in POC testing for multiple analytes. Often, the visual reading

Table 1

SARS-CoV-2 Test	Format	Sensitivity	Specificity	Limit of detection	Duration	Comments and additional references
Abbott ID NOW from Abbott Diagnostics Scarborough, Inc	RT-PCR	80%	100%	64 copies/mL	5-13 min	May not detect weakly positive samples https://doi.org/10.1016/j.jviromet.2021.114293
BioFire FilmArray,	Nested multiplex PCR; microfluidic chip technology	PPA 97%	NPA 100%	50 copies/mL	60 min	https://www.frontiersin.org/articles/10.3389/fmicb.2022.854209/full FDA approval for marketing
Cue COVID–19 Test	Isothermal nucleic acid amplification	91.7%	98.4%	NR	20 min	https://pubmed.ncbi.nlm.nih.gov/33571863/
Detect Covid–19 Test	Isothermal nucleic acid amplification	PPA 90.9% (30/33);	NPA 96.2% (76/79)	NR	60 min	https://detect.com/products/covid-19-test
Mesa BioTech Accula SARS–CoV–2 from Mesa BioTech	RT-PCR--LFA	Overall agreement 84% PPA 68%	NR	NR	30 min	False negatives may be due to low viral load samples
QIAstat–Dx	Multiplex PCR	100% PPA 85%	97% NPA 100%	1000 copies/mL	60 min	https://pubmed.ncbi.nlm.nih.gov/32341142/ https://www.frontiersin.org/articles/10.3389/fmicb.2022.854209/full
Roche Diagnostics Cobas 6800 SARS–CoV–2 test, Roche, from Molecular Systems, Inc.	RT-PCR	95.8%	NR	NR	96 results, 180 min	FDA approved; https://www.nsmedicaldevices.com/news/roche-cobas-sars-cov-2-qualitative-pcr-test/
Visby COVID–19 from Visby Medical Inc	RT-PCR	95%	100%	500 copies/mL	30 min	https://doi.org/10.1016/j.clinbiochem.2021.11.007
Xpert Xpress SARS–CoV–2 from Cepheid	RT-PCR	>99%	>99%	8.26 copies/mL	45 min	

LFA, lateral flow assay; NPA, negative percent agreement; NR, not reported; PPA, positive percent agreement; RT-PCR, reverse transcription polymerase chain reaction

Table 1: *FDA tests for detection of SARS-CoV-2 that have received emergency use authorization*

involves a color change due to aggregation of the Au nanobodies (AuNPs).

Visual inspection

Visualization includes turbidity changes, fluorescent dye methods or colorimetry that uses pH-sensitive dyes. Since results can be read by naked eye, advantages include no requirement for expensive equipment (eg, readers), intuitive results, and suitable for real-time detection at POC. However, the sensitivity of visual detection is lower than fluorescence detection.

Combining smartphone imaging with sensing platforms can provide a more objective result and improve the quantification of visual results, thereby increasing detection accuracy.

CRISPR/Cas detection

Two approaches with CRISPR/Cas are being investigated for POC testing.

Although the traditional SHERLOCK CRISPR method involves 2 separate reaction steps, Joung et al combined CRISPR-mediated detection steps with LAMP to yield a single step reaction that did not require sample extraction. The commercial LFA test strip can be completed in 1 hr and had a detection limit of 100 copies. [https://pubmed.ncbi.nlm.nih.gov/32511521/]

Cas12 is an RNA-guided DNA endonuclease that targets single-stranded DNA. Viral RNA is reverse transcribed into DNA and Cas12a is activated by the target sequence after isothermal amplification. Cas12a cleaves the ssDNA reporter and releases the fluorescent signal. This strategy can obtain sensitive and specific DNA detection while using an automated high throughput format. CRISPR can also be combined with LFA to generate real-time detection for home and POC testing.

Current POCT tests for SARS-CoV-2

FDA has given emergency use authorization (EUA) for the following tests:

- Abbott ID NOW
- BioFire FilmArray
- Cue COVID–19 Test
- Detect Covid–19 Test
- Mesa BioTech Accula SARS–CoV–2
- QIAstat–Dx
- Roche Diagnostics Cobas 6800 SARS–CoV–2 test
- Visby COVID–19 from Visby Medical Inc
- Xpert Xpress SARS–CoV–2 from Cepheid

The characteristics of these tests are summarized in Table 1. Most use RT-PCR technology as a platform and some have combined it with additional strategies for detection such as LFA. Several of the tests have recently gained FDA approval. The sensitivity of most tests ranged from 80% to 99%. Specificity was often higher. The duration of the assays ranged from 13 min to 180 min. The limit of detection varied widely, from <9 copies/mL to 1000 copies/mL.

Current POCT in China

The Sansure iPonatic, the 2019 novel coronavirus nucleic acid detection kit of Shanghai Toujing Life Technology Co., Ltd, uses one step sample lysis, DNA amplification, magnetic beads to extract the nucleic acid during the detection phase, followed by analysis of results. Because the magnetic beads need to be manually loaded, the test should be performed in a standard laboratory setting. The following processes are automatically completed: cracking (opening lid), magnetic bead extraction, DNA purification, DNA extraction, and isothermal amplification.

Quality assurance

Because detection of SARS-CoV-2 requires handling of human samples, the tests should be carried out in biosafety level 2 laboratory with personnel wearing appropriate protective gear (eg, gloves, masks, gowns). To avoid contamination of the different steps, three separate bench spaces with clean air should be set up for (1) reagent preparation area, (2) specimen preparation area (eg, A2-type biosafety cabinet), and (3) amplification area. In addition to using an approved, highly sensitive test, the laboratory needs to establish standard operating procedures in accordance with the manufacturer's instructions. As expected, the laboratory personnel should be trained and should run a control run for precision and reproducibility, preferably each day before handling human specimens.

Interview with Dr. Lisa Fitzpatrick,

Western Europe Diagnostic & Regulated Materials Marketing Manager

Can you introduce yourself for the reader.

My name is Lisa Fitzpatrick, I have been working at Merck KGaA, Darmstadt, Germany for over 20 years. My background is in the more regulated areas of science – such as environmental chemistry and more recently in diagnostic testing.

Why is integrity of the test sample so important to the effectiveness of a lateral flow test?

For any test to be valid, it needs to differentiate between positive and negative results effectively and efficiently. During validation, various sample matrices are tested, and the results are analysed. The validation test samples should be as close as possible in composition to the proposed clinical samples. In addition to this, sample collection and processing i.e. running the test, should aim to minimize changes to the sample material. Unnecessary changes can lead to false results – positive or negative - or an invalid data point.

What considerations regarding sample sources are key in the development process?

Sample preparation should always be as minimal as possible – keeping the sample integrity is key to accurate results. Developing a test that is robust in the face of many different sample types e.g. whole blood & plasma, urine, saliva, tissues etc. is always going to be a challenge, but one that needs addressing right at the start of assay development. Even simple modifications such as dilution, also come with their challenges as this can potentially reduce the analyte of interest to below the LOD of the assay. More complex sample preparation, such as extraction, increase the risk of sample contamination, potentially removing any analyte of interest, thus, reducing the accuracy and precision of the assay.

The European Union has recently enacted new *in-vitro* diagnostic regulations. How do these impact the development process for these tests? And how do they correlate with FDA regulations?

IVDR has been a big change for the entire IVD industry. There are certainly more responsibilities placed on the manufacturers of IVDs, both pre and post market launch of any IVD. Integrity and transparency of supply chain is also strongly emphasised; which directly impacts the selection of raw materials and services right from assay concept through to clinical use. Any manufacturer wishing to take advantage of the European market, must produce their devices in accordance with IVDR, irrespective of their manufacturing geography.

On the whole, the FDA IVD and EU IVDR are aligned, but there are a couple of minor differences between the two. This is mainly around terminology on device classification (Class I (low risk), Class II (moderate risk), Class III (high risk) for FDA IVD and Class A (lowest risk), B, C, D (highest risk) for IVDR), although both systems of classification are risk based. The other area of slight difference is around PMS (post market surveillance), and is more reactive for FDA IVD, whereas IVDR demands a more formal recording system of proactive reporting.

Where do you see this field headed in the next 5-10 years in terms of advances and need given the focus on emerging infectious diseases?

What the COVID pandemic has shown us, is that infectious diseases (bacterial or viral) can come from *anywhere*. It highlights the need to monitor and assess infection potential from zoonotic (and reverse zoonotic) sources, as well as being mindful of the antimicrobial resistance issues facing infection treatment, and a need to use the most appropriate course of action. The One Health approach fosters this closer collaboration between all the factors affecting health at a global level. Moving forward, fast and accurate differentiation of viral and bacterial sources of infection will be of paramount importance, to mitigate the risks of ineffective medication, and ensure that any infectious disease has a suitable therapy.

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Interview with Dr. Matthew Coussens,

IVD Assay Development Training Manager

Can you introduce yourself for the reader.

My name is Matt Coussens. I have been part of MilliporeSigma's customer training program for the last 13 years. It has been my pleasure to travel the globe teaching fellow scientists such techniques as qPCR, NGS, RNAi, genome editing, and lateral flow test development.

What are the key components to a lateral flow assay?

Commonly available lateral flow tests typically rely on a "sandwich" type of immunoassay. Many variations are possible, but they all have in common the formation of a complex between a detector particle that is free in the sample stream and a capture reagent(s) that is bound to the membrane at the test and control lines.

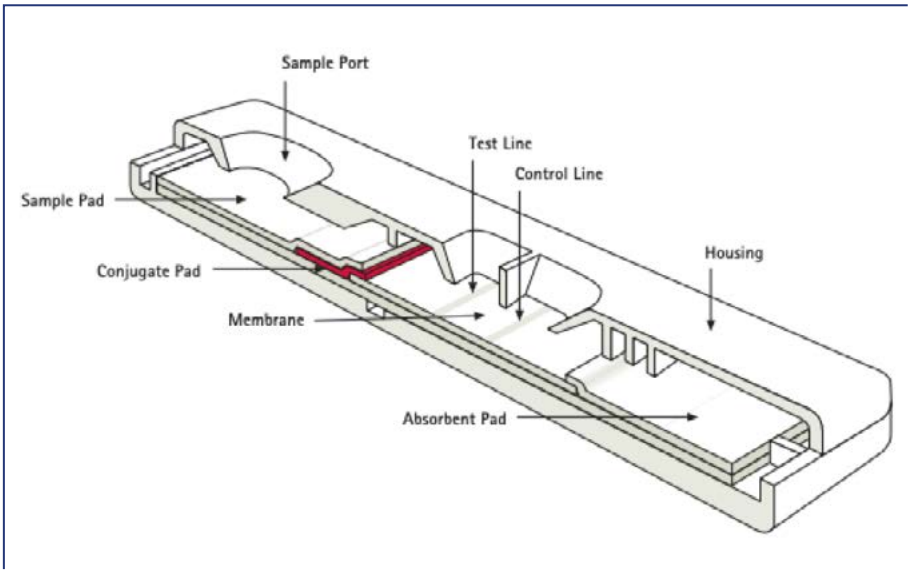
For antibodies selection, the rise of recombinant antibodies is driving the development of new lateral flow tests. What are recombinant antibodies and what do you feel are their strengths and weaknesses?

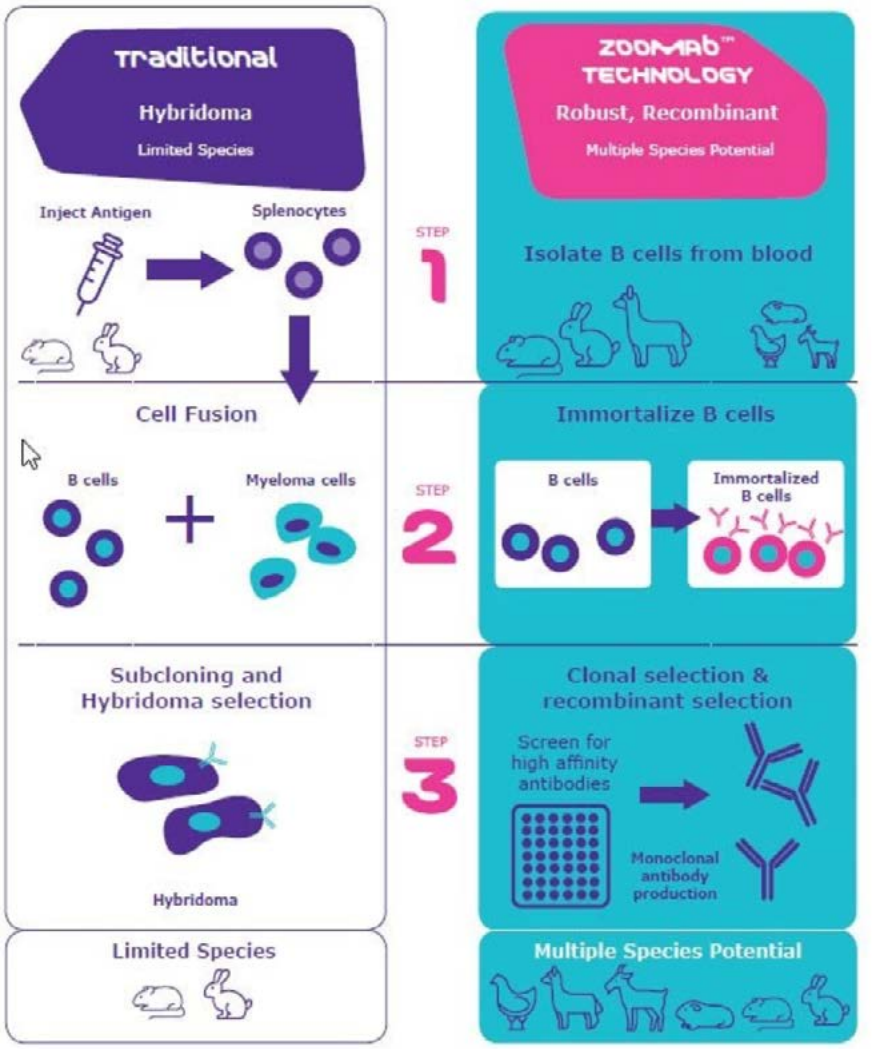
Recombinant antibodies are monoclonal antibodies that are generated by cloning antibody genes into expression vectors and do not involve the use of hybridomas. These antibodies can be cloned from any species using suitable oligonucleotide primers. With this technology, the problems of cell line drift, antibody expression variations, and antibody sequence mutations associated with classical hybridoma production and storage can be avoided.

Recombinant antibodies represent an entirely new generation of monoclonal antibodies that offer the superior specificity and affinity of a monoclonal antibody, the reproducibility of recombinant technology, and greener alternatives including sustainability. Furthermore, each and every recombinant antibody produced by us is done so without the harm or sacrifice of animals. The recombinant expression system eliminates the need for arduous cell fusion and hybridoma construction, while providing greater specificity, affinity, and reproducibility than monoclonal counterparts.

Do you have any recommendations or good practices for deciding on what type of detection chemistry to use in your design?

Various types of detector reagents can be used for the visualization of a signal. The most commonly used materials in commercially available tests are latex beads, colloidal gold particles, and Estapor® microspheres. Other possibilities include enzyme conjugates, other colloidal metals, fluorescent particles, and magnetic particles. One of the most important features of the particles is that the population is monodisperse with consistency of size and spherical shape. When a test is run, the particles are required to move through the torturous pore structure of the membrane. Smaller particles move faster than larger particles. Particle preparations with different size and shape distributions will move through the membrane differently. This can lead to differences in apparent sensitivity and specificity, even when all other components of the test are identical. Specific methods for preparing various particles





and conjugating antibodies to them can be found in the literature. Commercial sources are also available. Since the conjugated detector particles are one of the key reagents in the finished test strip, methods for their preparation and handling should be fully validated. Similarly, relevant quality control methods need to be established.

If they are purchased from a vendor, they should come with specifications on the attributes relevant to performance in lateral flow tests.

Where do you see this field headed in the next 5-10 years in terms of advances and need given the focus on emerging infectious diseases?

With their rapid adoption for use during the COVID pandemic, the general public has become very familiar with the process of running and interpreting the results of a standard lateral flow test. Because of this, I think that we will see a proliferation in direct-to-consumer qualitative lateral flow tests for a variety of purposes. Similarly, we will see an uptick in the number of quantitative tests and readers coming into the market targeted for use in healthcare settings, schools, and other environments where trained professionals can run and accurately interpret results. Companion animals, agriculture, and food safety are additional markets where we are already seeing an increase in the demand of suitable lateral flow tests.

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Further Reading

Accelerating Time to Market Using Custom Lateral Flow Membranes
https://www.sigmaaldrich.com/technical-documents/technical-article/clinical-testing-and-diagnostics-manufacturing/ivd-manufacturing/oem-custom-lateral-flow-membranes

Estapor® europium microspheres: improved lateral flow applications
https://www.selectscience.net/application-articles/estapor-europium-microspheres-improved-lateral-flow-applications/?artid=58707

IVD lateral flow – sample, conjugate, and absorbent pad basics
https://www.sigmaaldrich.com/technical-documents/technical-article/clinical-testing-and-diagnostics-manufacturing/ivd-manufacturing/pads-chemistries-selections-specifications-and-conjugates

Making IVD lateral flow test strips for analytes
https://www.sigmaaldrich.com/technical-documents/technical-article/clinical-testing-and-diagnostics-manufacturing/ivd-manufacturing/getting-started-with-ivd-lateral-flow

Sensitivity of lateral flow diagnostic assays with ultra-bright gold nanoshell reporters
https://www.sigmaaldrich.com/technical-documents/technical-article/clinical-testing-and-diagnostics-manufacturing/ivd-manufacturing/sensitivity-lateral-flow-diagnostic-assays

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