

Lateral Flow Assays in Infectious Disease Diagnosis

Hans R. Boehringer* and Brendan J. O'Farrell

BACKGROUND: Lateral flow immunoassays are widely used as diagnostic tests in many applications in human and other diagnostic areas. Assays for human applications have been commercially available since the 1980s and initially were primarily used to identify pregnancy by measuring human chorionic gonadotropin in urine and serum/plasma.

CONTENT: The first infectious disease lateral flow assays were commercialized in the late 1980s identifying the presence of Group A *Streptococcus pyogenes* collected with throat swabs; innumerable other applications followed in the intervening decades. The severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) pandemic has brought a vast number of new assays for which emergency use authorization (EUA) has been requested in the USA. These assays have been designed for detection of the antibody response to an infection and viral antigens in respiratory samples. In view of the onslaught of new tests, this review will focus on the use of rapid lateral flow immunoassays for infectious diseases. Principles of lateral flow assays and approaches to the production of high-sensitivity point-of-care assays are presented. Market trends, customer requirements, and future directions of lateral flow assay technology and its applications in the infectious disease diagnostic space are discussed.

SUMMARY: Lateral flow immunoassays play an important role in infectious disease diagnostics. Advancements in technology have led to improved performance of these assays and acceptance by professional users. With the advent of the SARS-CoV-2 pandemic, the market has reached new levels requiring hundreds of millions of tests per year for professional and even home use.

Lateral Flow Assay Principles

Traditionally designed lateral flow assays (LFAs) are composed of a variety of materials, each of them serving

one or more purposes. Those components are mounted on a backing card using pressure-sensitive adhesives. There are numerous publications describing the basic components, architecture and functions of LFA strips, including Brown (1) and O'Farrell (2).

In general, to perform an LFA test, the sample is added to the proximal end of the strip, where a “sample application pad” supports sample treatment, making it compatible with other test elements. Treated sample then migrates onto the “conjugate pad,” containing immobilized conjugate, typically made of nanoparticles (e.g., colloidal gold, colored, or fluorescent latex, colored cellulose) conjugated to either antibodies or antigens. When the sample rehydrates and mobilizes the dried conjugate, analyte in the sample interacts with the conjugate as both migrate into the next section of the strip, the reaction matrix. Typically, the reaction matrix is a porous nitrocellulose membrane onto which the other biological components of the assay have been immobilized. These are generally proteins, either antibodies or antigens, that have been dispensed and dried in bands on the membrane where they capture the analyte and conjugate. Any excess reagents move past the capture lines and accumulate in the absorbent pad. Results are interpreted on the nitrocellulose membrane as the presence/absence of lines of captured conjugate, interpreted either by eye or by using a reader (1).

It is imperative diagnostic assays provide accurate information to decision makers to have value to end-users. The ability to provide accurate information for diagnostic technologies ranges from highly accurate methods requiring infrastructure and centralized laboratories to less accurate technologies that can be used in decentralized environments, and point-of-care or even home settings. LFAs have been associated with the latter environments; the technology was considered a cheap solution to simple problems. Recent advances in reagents, materials and manufacturing processes improved LFA performance in applications requiring higher accuracy and sensitivity while maintaining the advantages of the basic technology. These advances led to improved applications for LFAs in decentralized testing environments, medical, and others. Applications in the developed world have taken advantage of the improved performance to generate LFA performance equivalent to that available with complicated laboratory-based formats. These high-performance assays use lateral flow strips combined with specialized readers, sample handling devices, and

DCN Diagnostics, LLC, Carlsbad, CA, USA.

*Address correspondence to this author at: 6354 Corte del Abeto, Suite B, Carlsbad, CA 92011, USA. E-mail hboehringer@dcndx.com.

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cartridges with onboard functionality—essentially creating laboratory analyzers based on the lateral flow format. Many other lateral flow applications fall in between the 2 extremes, demanding high performance and ease of use, coupled with mobile technology for high-end field-based applications. In short, the LFAs, once the poor relative of laboratory-based tests, has evolved into a versatile technology capable of more than adequate performance at all points on the diagnostic continuum. As these improvements are integrated into LFAs there are, however, still many assays that have not reached that level of performance, including ultrasensitive thyroid-stimulating hormone (TSH) assays for example.

The significant evolution in LFA performance and capabilities occurring over the past decade has been achieved through continuous improvements in materials, reagents, manufacturing equipment, and process technologies, and from next-generation facilitative technologies (2). Among critical components with direct impact on sensitivity and specificity of LFA performance are recognition reagents, typically antibodies, which are used most often, as well as aptamers or affimers. While difficult to predict reagent performance in LFAs, affinity constants are the major defining parameters besides specificity. Because interactions between target antigens and capture reagents are limited to a few seconds, high on-rates are an important characteristic. Interactions between conjugate antibodies and antigens also occur quickly, although mixing time can last several minutes. High affinity antibodies generally provide better results, as their affinity constants can be in the picomolar range, while aptamers are in the nanomolar range, at least 1 or 2 orders of magnitude less strong. Recombinant or genetically engineered antibodies can be designed to achieve picomolar or better affinities, thus generating LFA sensitivities approaching amplified nucleic acid test levels. It is well documented that antibody-engineering approaches will improve affinities for both therapeutic and diagnostic applications (3). Boder and colleagues reported antibodies against fluorescein engineered to have femtomolar affinities (4), a 10 000-fold improvement over original antibody clones; others reported

affinity improvements to antibodies for steroids (5) and carcinoembryonic antigen (6).

Other components of LFAs that have led to large improvements in sensitivities (10–100-fold) over colloidal gold-based assays are detector particles. Advances in gold manufacturing, including covalent gold particles and nanoshells (nanoCompositex, San Diego, CA), can provide 5–10-fold improvements in analytical sensitivities. New particles, such as highly colored cellulose nanobeads, NanoAct™ (Asahi-Kasei Corp, Japan), have increased sensitivities up to tenfold in several assays (Table 1). The use of fluorescent particles, specifically europium (Eu) chelate-doped polystyrene latex particles, results in even greater analytical sensitivity improvements. Eu-latex based LFAs have 50–100-fold improvements over colloidal gold-based assays (internal data; DCN Diagnostics, Carlsbad, CA).

Nucleic acid diagnostic assays for infectious diseases, in contrast to LFAs, require expensive infrastructure, sophisticated equipment, and complex processes. Due to their superior performance and accuracies, they are often the gold standard methods against which LFAs are measured. In diagnostic infectious disease applications, nucleic acid detection is widely used, but requires amplification steps, including PCR or methods such as isothermal amplifications (7). Despite the generally superior performance of nucleic acid assays, the complex laboratory environment, long turnaround time, and higher costs to implement them are significant downsides. Recently, molecular diagnostic assays and lateral flow have intersected at the point-of-care, with lateral flow being used as the readout technology for nucleic acid testing (e.g., Mesa Biotech). Additionally, with the advent of CRISPR-based diagnostic testing, it is a natural next step to use the lateral flow platform to bring this highly sensitive and specific diagnostics to the point-of-care (POC); work in that regard is underway at several sites.

APPLICATIONS AND MARKET SIZE

In general terms, the LFA market can be categorized by application into veterinary diagnostics, food safety and

Table 1. High-sensitivity visual lateral flow assay (LFA)-colloidal gold vs. cellulose nanobeads based detection using same reagents and materials (internal data; DCN Diagnostics, Carlsbad, CA).

Analyte	Colloidal gold LFA: limit of detection	Cellulose nanobeads LFA: limit of detection	Limit of detection improvement
hCG	2.5 mIU/mL hCG	0.25 mIU/mL hCG	10-fold
Troponin I	250 pg/mL TnI	30 pg/mL TnI	8-fold
HIV p24 antigen	500 pg/mL p24	50 pg/mL p24	10-fold

environmental testing, drug development and quality control testing, and clinical/POC testing, which accounts for the largest share.

Factors driving growth in the clinical/POC testing market include rising population levels, chronic diseases prevalence, emergence of the Coronavirus pandemic in 2020, growing pressure to reduce healthcare costs, and increasing demand for patient-centric care; because of these factors the market is likely to exceed previously projected values. The projected additional growth in 2021 and onwards is mainly driven by the global severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) pandemic, requiring substantial volume increases for antigen LFAs in the USA and worldwide, especially in low- and middle-income countries (LMICs).

As a large fraction of the human LFA market, infectious disease lateral flow assays can be further differentiated into additional categories (8) such as respiratory tract infections (including influenza, SARS-CoV-2, respiratory syncytial virus, Strep A); mosquito-borne diseases (including malaria, dengue, zika); sexually transmitted diseases (chlamydia, syphilis, gonorrhea); tuberculosis; blood borne pathogens (HIV, hepatitis B, hepatitis C); and other infectious diseases.

The infectious disease market size for LFAs is complex, as various factors drive the annual sales and growth for individual markets. For example, infectious respiratory disease sales are typically seasonal and depend on the severity and spread among various populations and geographies. Performance of infectious disease LFAs is also driving the market, as competitive technologies, especially molecular diagnostics have captured a large market share. Clinical and analytical sensitivities in general are higher and represent better values in molecular assays, but LFA costs and assay time are still superior, thus supporting a large market for POC and home testing, and expansion into LMICs.

Predictions of market size and growth made prior to the SARS-CoV-2 pandemic put the global LFA market at \$8240 million by 2022, with a compounded annual growth rate of 8% and \$9650 million by 2025 (8). The biggest infectious disease markets with the highest growth rates were anticipated to be in tropical infectious diseases [malaria and tuberculosis (TB)], and in HIV and influenza.

The coronavirus disease (COVID) pandemic has vastly affected the anticipated evolution in this market. First, there is the substantial requirement for SARS-CoV-2 antigen and serological assays. Published company financial performance from 3 of the largest producers of rapid tests in the USA, Abbott, Quidel, and Becton Dickinson (BD), put combined sales from calendar Q4 of 2020 through the end Q1 2021 at more than \$5.5 bn. Along with sales comes significant build-up of infrastructure, which will fuel future growth of their

platforms and menu of products. For example, BD announced that it had deployed more than 70 000 Veritor™ reader platforms by the end of Q1 2021. This level of penetration into small laboratories, pharmacies, and ultimately homes will drive an entirely new evolution in the LFA market. Combined with recent entrants to the lateral flow development and manufacturing ecosystem such as Luminostics Inc. (Milpitas, CA), Celltrion USA Inc. (Jersey City, NJ), Ellume Ltd. USA (Valencia, CA), and Salofa Oy (Salo, Finland), all of which have EUA-approved antigen detection assays, high levels of grant funding through programs such as NIH RADx, and public interest in self-testing and home-testing, lateral flow markets are poised for significant short- to mid-term growth. Companies that have EUA-approved tests are listed on FDA-EUA websites (9, 10) and summarized in Table 1 in the online Data Supplement.

The SARS-CoV-2 pandemic has led to capacity expansions throughout the entire supply chain, from raw materials to reagents to manufacturing capacity and finished goods. However, it also adversely affected other major global health initiatives and their related diagnostic markets. A recent report by *The Global Fund* describes the impacts on testing for HIV, malaria, and TB in global developing-economy markets, based on a sampling from 24 African and 7 Asian countries. In the period April–September 2020, HIV testing numbers decreased by 41%, TB testing and screening fell by 52%, and malaria testing and diagnosis by 56% (11).

The influenza season, as reported by the CDC (12), and testing markets were severely disrupted in 2020 and it remains unclear as to what impact population behavioral changes will have on future influenza patterns. Strong growth is expected in requirements for differential diagnosis between COVID and other respiratory infections, so combination tests including Flu A/B (influenza A and B), SARS-CoV-2, and others such as respiratory syncytial virus may show continued strong growth.

In short, the current climate makes it virtually impossible to accurately predict the size of the LFA market over the next several years. However a few broad conclusions are possible: the LFA market is positioned for continuing strong growth; COVID testing markets will decrease over time but it will likely remain the largest sector for several years, as new variants and use cases emerge and the home-testing market matures; non-COVID testing markets in traditional applications will recover as global supply capacity increases and demand in developing economies recovers over time; new appreciation for the benefits of lateral flow technology in the public mind and acceptance of its capabilities and benefits in the regulatory and clinical environment will lead to substantial opportunities for its adoption in

nontraditional applications; this will be supported by the expanded supply chain and infrastructure, and by broadly accepted advances in reader technology, digital result interpretation, and data reporting.

ADVANCES AND FUTURE PERFORMANCE OF INFECTIOUS DISEASE LATERAL FLOW ASSAYS

This section describes some of the relevant technical advances that will affect LFA performance and affect rapid diagnostic testing in POC settings. A detailed review of the different aspects of technology improvements has been described by O'Farrell (2), and the most recent and current approaches are discussed next in this mini review.

Innovation in each of the key components of LFAs is required to improve performance and permit their effective application at both the low resource end of the spectrum and in higher-end applications in developed world environments. The key components and technologies that have to be addressed in development of POC diagnostic devices for any application are: recognition and signal generation, readout and signal-transduction technologies, sample collection and handling (e.g., concentration and preparation), and device design. It is critical that these improvements are then integrated into carefully controlled manufacturing processes to produce highly sensitive, reproducible, quantitative, and, if needed, multiplexed systems.

Finally, among the most critical improvements that will be demanded going forward will be the ability to objectively interpret results with high fidelity, and collect and report data from rapid tests through ubiquitous digital means.

Recognition and signal generation: The recognition elements in LFAs are typically antibody-antigen binding reactions or antigen binding to alternate recognition molecules including aptamers or affimers. The binding constants these molecules to antigens are critical parameters that need consideration when selecting antibodies. Affinity constants for antibodies are often 10–100 fold stronger than for aptamers or affimers. Genetically modified antibodies with even higher affinities can conceivably be developed and further improve binding reactions for highly sensitive LFAs. Additionally, inclusion of the high affinity biotin–avidin reaction in LFAs has often improved assay performance. This approach can also be applied to aptamers, affimers, or recognition reagents, and can improve their performance. Screening and selection criteria for recognition molecules has been discussed in detail by O'Farrell (2).

Readout and signal transduction: Visual POC immunoassays were originally designed to be qualitative yes/no tests interpreted by the user without the use of a reader. Most LFAs are still based on subjective interpretation by the user, which is a major limitation for those

assays. It limits the technology to qualitative applications; also, data loss and user error in interpretation and result transcription are major challenges, especially in a POC or home-testing environment. Thus, developing LFAs with an integrated reader system is often a required product design specification for next-generation assays. These reader systems must be capable of interpreting results with high fidelity and transmitting them to health authorities. A variety of technical, financial, and regulatory considerations must be considered in the choice of reader systems and in the design of the assay itself.

Choice of label is one key consideration in the overall system design that can affect reader selection and overall system performance capabilities. Various visual particles and their performance in LFAs and fluorescence-based assays, often using europium labels for applications requiring high analytical sensitivity and quantification, are becoming more commonly used and were discussed previously.

Commercial readers for LFAs are widely used in today's market and are expected to become more ubiquitous over the next several years. Multiple categories of reader system are used, depending on application environment and regulatory requirements of that application. Broadly, they can be categorized as: in-cassette or disposable readers such as the Clearblue pregnancy test system with integrated digital reader; custom, low-cost consumer-facing readers produced for specific applications, e.g., NIMA (San Francisco, CA), Inne by Feral (Berlin, Germany); fully validated and calibrated benchtop clinical readers e.g., Veritor (BD), SOFIA (Quidel); and cellphone cameras coupled with apps. Behind many of these reader systems are dedicated OEM reader suppliers such as Dialunox (Stockach, Germany), Planet Innovation (Australia), IUL S.A. (Spain), and DCN Dx (Carlsbad, CA), and app suppliers such as Abingdon Health (UK) and Novarum/BBI (UK). The cell phone and app segment are additionally supported by custom software suppliers including Bond Digital Health (UK), who support the app design and the back-end data management component. Phone-based apps are viewed as key to broad-based adoption of POC rapid testing. The app can fulfill multiple functions including improving user-performance through step-by-step instructions, improving image capture and result fidelity through the use of image analysis, and allowing for real time reporting and databasing of results for use by regulators and health authorities.

Sample collection. Sample collection is critical for performance of LFAs in any setting, but especially in POC testing. The pandemic has illustrated the importance of proper sample collection for overall test performance,

especially when used by minimally or untrained POC users. Sample collection, treatment, and delivery to the LFA must be simple, robust, fail-safe, and ideally integrated with the device to reduce user errors and variability. Where integration is not possible, user studies are critical to develop sampling methods that can be used in those environments. A good example would be the CDC nasal swab collection guidelines for the collection of SARS-CoV-2 samples for POC and home use tests (13). Studies have shown that self-collected samples perform equally well in COVID-19 tests as clinician-collected samples (14). Oral swabs are another sample type that can be integrated with infectious disease LFAs in POC environments. Variability of the sample matrix is an important factor in assay variability and needs to be considered when using oral swab samples. Fingertick blood samples are another desirable sample type for serology assays; the challenges of collecting and delivering those samples have been discussed and described by O'Farrell (2).

Device design. User-centric design of rapid diagnostic devices has historically not received the attention it deserves. The focus was typically on cost, meaning that most people think of cheap, white plastic when they visualize a lateral flow test. Usability, however, is the key to adoption and proper performance of the assay, and is a first-tier design issue. Design encompasses all aspects of the user experience, from taking the device out of the packaging, acquiring the right sample, delivering it to the test, running the device, and interpreting and reporting the results.

The design of the test device/cassette is critical for the LFA performance and requires that the cassette be custom designed for the actual test strip and application. In most high-fidelity applications, taking an off-the-shelf cassette available on the open market is not a viable option. Designing custom lateral flow cassettes can result in important performance benefits, including production of assays with low variability, allowing quantitation, ability to integrate the assay and cassette with a reader system, controlling the sample adsorption to the test strip, and optimizing flow. Controlling sample flow through proper cassette design is a feature critical to LFAs, assuring uniform flow, rehydration of dried reagents, mobilization of the particulate conjugate, and eventual transfer to the nitrocellulose membrane. Well-designed pressure points on material overlaps are key features to ensure even flow through the test strip. Additional consideration needs to be given to cassette-closure as critical strip components will be crushed and damaged if too much pressure is applied. The shape/angles of the read window are critical for cassettes used in a reader to avoid shadows when illuminating the test.

Future Direction for Infectious Disease LFAs

It is difficult to predict how the infectious disease lateral flow market will evolve in the next 5–10 years, as many factors are changing rapidly. Who would have thought that rapid antigen lateral flow tests for the detection of coronaviruses would be required worldwide in the 100's of millions for POC and home testing in 2021–22? In addition to the unexpected need to detect emerging infectious disease pathogens, major shifts are underway in the clinical/POC market place, including the way in which products are distributed, bought and paid for, in how these assays are regulated, and how new markets such as LMICs have needs for large volumes of these tests.

Technology-wise, the focus of assay developers will likely stay on the implementation and continuous improvements of test strips to achieve better sensitivity, reproducibility, and quantification. Multiplexing for panel testing such as respiratory panels, sexually transmitted disease panels, etc. to expand test menus will also be a focus for many. The need for home testing and consumer diagnostics will continue to expand device designs to include integrated features that are intuitive for minimally or untrained users. POC molecular diagnostic tests represent an important emerging segment of the in vitro diagnostic market, including CRISPR-based assays (15). As molecular diagnostic assay technology has evolved, companies have developed fully automated systems, and a growing number of commercially available products have been categorized as moderate complexity tests under the CLIA regulations. This categorization makes it possible for the tests to be moved from the central laboratory to locations that are closer to patients. While achieving moderate complexity classifications represented an important milestone, the ultimate goal is to develop and commercialize tests for POC settings that are so automated and easy to use that they can be granted a CLIA waiver. Many emerging diagnostic companies are pursuing this goal. These emerging tests typically use an amplification step (PCR or another amplification technology) as the sample combined with a LFAs for detection of the amplified nucleic acid products (NALF). NALF has been used successfully in increasing numbers of systems, and methods for developing and manufacturing these devices are relatively simple (16, 17). Several NALF systems/platforms have been commercialized recently, including AmpliVue (Quidel Corp., San Diego, CA), Amodia (Amodia Bioservice GmbH, Germany), HybriDetect (Milenia Biotec, Germany), and DCNovations NALF kit (DCN Diagnostics, Carlsbad, CA). Based on recent development and success of these systems, it is expected that NALF will be a significant tool in coming years by combining amplification and lateral flow steps in a single

Table 2. Key specifications of future high-performance lateral flow assays (LFA).

	High-performance visual lateral flow	High-performance fluorescent lateral flow	Nucleic acid lateral flow
Detection principle	High-performance colored nanoparticles (incl. covalent gold; gold nanoshells; cellulose nanobeads)	Europium-chelate latex; fluorescent dye latex; quantum dots	Visual nanoparticles including colloidal gold; colored latex; cellulose nanobeads
Interpretation	Visual or reader	Reader-based only	Visual or reader
Quantitative	Yes, possible	Yes, possible	No
Sample	All types	All types	Amplification products only
Sample processing	Application dependent	Application dependent	Required
Sensitivity	μ M; nM; high pM	nM; pM, fM	N/A, amplification step dependent
Assay time	5-30 min test time	5-30 min test time	Amplifying reaction time + 5-10 min LFA time
Multiplexing	Yes; 4-8 per test strip	Yes; 4-8 per test strip	Yes; limited by available primer tags

device. Table 2 shows a comparison of key features of future LFA technologies.

The World Health Organization developed the ASSURED criteria (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end-users) for the development of future diagnostic assays more than a decade ago. The lateral flow platform remains the best available assay format that addresses those criteria. It demonstrates flexibility for many applications, and meets performance specifications approaching levels of much more complex technology systems. Lateral flow is not a product in and of itself, but rather the engine that drives billions of tests annually, around which products are designed to address any market space. Despite many years of efforts and billions of dollars of expenditure, there is still no other broad-based open platform that can approach lateral flow in its flexibility and applicability, speed of development, cost, performance, and supply chain robustness. Thus, it is very likely that the LFA platform will have major market shares in infectious disease POC testing for years to come.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: SARS, severe acute respiratory syndrome; CoV-2, coronavirus 2; LFA, lateral flow assay; TSH, thyroid-stimulating hormone; CRISPR, clustered regularly interspaced short palindromic repeats; POC, point-of-care; LMIC, low- and middle-income country; TB, tuberculosis; COVID, coronavirus disease; CDC, Centers for Disease Control; NALF, nucleic acid lateral flow

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