ACS NANO

www.acsnano.org

PERSPECTIVE

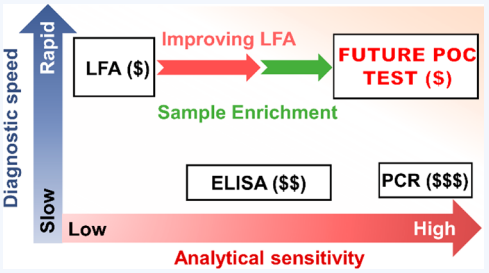
Ultrasensitive and Highly Specific Lateral Flow Assays for Point-of-Care Diagnosis

Yilin Liu, Li Zhan, Zhenpeng Qin, James Sackrison, and John C. Bischof*

Cite This: https://dx.doi.org/10.1021/acsnano.0c10035 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

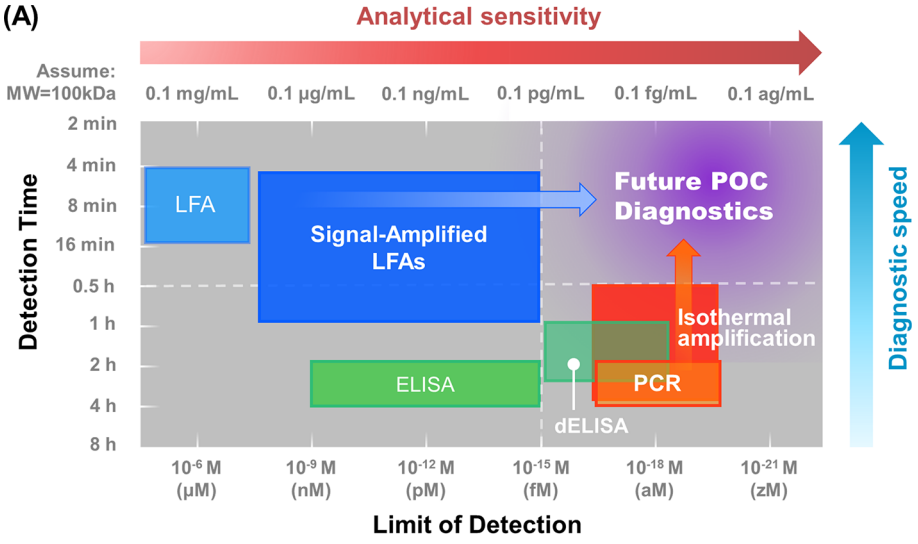
ABSTRACT: Lateral flow assays (LFAs) are paper-based point-of-care (POC) diagnostic tools that are widely used because of their low cost, ease of use, and rapid format. Unfortunately, traditional commercial LFAs have significantly poorer sensitivities (μM) and specificities than standard laboratory tests (enzyme-linked immunosorbent assay, ELISA: pM−fM; polymerase chain reaction, PCR: aM), thus limiting their impact in disease control. In this Perspective, we review the evolving efforts to increase the sensitivity and specificity of LFAs. Recent work to improve the sensitivity through assay improvement includes optimization of the assay kinetics and signal amplification by either reader systems or additional reagents. Together, these efforts have produced LFAs with ELISA-level sensitivities (pM−fM). In addition, sample preamplification can be applied to both nucleic acids (direct amplification) and other analytes (indirect amplification) prior to LFA testing, which can lead to PCR-level (aM) sensitivity. However, these amplification strategies also increase the detection time and assay complexity, which inhibits the large-scale POC use of LFAs. Perspectives to achieve future rapid (<30 min), ultrasensitive (PCR-level), and "sample-to-answer" POC diagnostics are also provided. In the case of LFA specificity, recent research efforts have focused on high-affinity molecules and assay optimization to reduce nonspecific binding. Furthermore, novel highly specific molecules, such as CRISPR/Cas systems, can be integrated into diagnosis with LFAs to produce not only ultrasensitive but also highly specific POC diagnostics. In summary, with continuing improvements, LFAs may soon offer performance at the POC that is competitive with laboratory techniques while retaining a rapid format.



Although infectious diseases have always posed global threats, there is no clearer current example of the need for inexpensive and high-performing (*i.e.*, low rates of false negatives (FNs) and positives (FPs)) point-of-care (POC) diagnostics than with the global pandemic of SARS-CoV-2 (*i.e.*, COVID-19). As a single serological antibody or antigen test can only indicate past or recent exposure to SARS-CoV-2, multiple and broad testing throughout the population will be needed to identify "hot spots" and to control the disease effectively.[1−5] In an ideal situation, at-risk individuals would be tested regularly (*i.e.*, weekly or daily) to enable timely isolation and to minimize virus transmission among the community.[1−5] Unfortunately, this need cannot be met using the current primary diagnostic tools, such as reverse transcription polymerase chain reaction (RT-PCR). Although RT-PCR has excellent sensitivity and specificity, it cannot be used as a POC test because this method requires trained staff in laboratories equipped with specialized thermal cycling equipment and strict environmental conditions to prevent contamination.[6−8] In addition, the long turnaround time (hours to days) and high cost of RT-PCR (100−200 USD per COVID-19 swab test) compared to other rapid (<15 min) diagnostic tools, such as lateral flow assays (LFAs; <$50 per COVID-19 swab test),[9,10] restrict its deployment in POC settings. These costs can be expected to drop further because other commercialized LFAs, such as human chorionic gonadotropin (pregnancy) LFAs, are <$1 per test.

Because LFAs are arguably the cheapest, fastest, and easiest to use paper-based POC tests,[11−15] they exhibit promise as a tool for achieving global pandemic control by enabling the rapid screening of infections. In addition to the detection of SARS-CoV-2, LFAs have also been widely applied in biomedicine, food contaminant and toxic chemical detection, and environmental monitoring.[12] In biomedical diagnosis, an important advantage of this technology is that it enables the decentralization of laboratory testing to POC sites. Some important examples of LFAs are those used for the rapid diagnosis of influenza,[16,17] *Streptococcus*,[18,19] and many other viral and bacterial infections.[20−22] The Centers for Disease

(A)



(B)

LFAs	Limit of Detection (M)	Time (min)
Electrochemical	10^{-9} ~ 10^{-15}	10 ~ 60
SERS	10^{-9} ~ 10^{-15}	15 ~ 30
Fluorescence	10^{-8} ~ 10^{-15}	10 ~ 60
Chemically enhanced	10^{-9} ~ 10^{-14}	5 ~ 60
Photothermal	10^{-7} ~ 10^{-13}	10 ~ 25
Magnetic	10^{-7} ~ 10^{-13}	10 ~ 30

Figure 1. Comparison of the analytical sensitivity and diagnostic speed of signal-amplified lateral flow assays (LFAs) with those of other diagnostic tools. (A) Comparison of signal-amplified LFAs with emerging isothermal nucleic acid amplification diagnostics, digital enzyme-linked immunosorbent assay (dELISA), and commercial diagnostic tools. (B) Estimated limit of detection ranges and detection times for different signal-amplified LFAs. A detailed literature summary of the data provided in (B) is shown in Table S2. The conversion of units between g/mL and molarity (M) is achieved with the molar weight of the target analytes. Chemically enhanced LFAs include LFAs that use novel labels and enhancing LFA reagents. Photothermal LFA methods include thermal contrast,[43,44] photoacoustic imaging,[45,46] photothermal laser speckle imaging,[47] and thermal photonic lock-in imaging.[48] SERS: surface-enhanced Raman scattering; PCR: polymerase chain reaction; POC: point of care.

Control and Prevention estimates that 9−45 million influenza infections occur in the United States each year, leading to 140,000−810,000 hospitalizations and 12,000−61,000 deaths per year.[23] Rapid influenza LFA testing provides results within minutes, thus enabling timely clinical decisions and medical treatment. This rapid diagnosis, in turn, provides enormous healthcare benefits, including slowing disease transmission, reducing the number of hospitalizations, decreasing downstream treatment costs, and minimizing antibiotic use.[16,18,19,24−26]

However, traditionally built commercial LFAs have several limitations, including poorer sensitivity (more FNs) and lower specificity (more FPs) than laboratory tests (see the sensitivity comparison in Figure 1A). These limitations also hinder the diagnosis and transmission control of diseases such as SARS-CoV-2,[22] influenza,[17,27] *Streptococcus*,[18,19] and HIV[28,29] by LFAs, thus maintaining the requirement for diagnostic confirmation by more complex laboratory tests such as PCR and enzyme-linked immunosorbent assay (ELISA). For example, even though commercial SARS-CoV-2 LFAs are available, the clinical assay accuracy was much lower (positive predictive value: 11%−50%) than the claimed sensitivity (87%−97.5%) and specificity (100%), which limited the impact that LFAs have on pandemic control and management.[22] Similar issues have also been reported elsewhere.[30,31]

To address these limitations, extensive efforts have been invested into improving the sensitivity and specificity of LFAs to achieve more accurate and higher-performing POC tests. Two important strategies for improving the sensitivity include assay improvement[12,13,32] and sample enrichment. As summarized in Figure 1A, signal amplification can improve the detection sensitivity by several orders of magnitude, which enables LFAs to achieve ELISA-level sensitivities. Further, sample preamplification with an LFA readout provides access to PCR-level sensitivity. Unfortunately, many of these techniques for improving LFA sensitivity require longer assay times (Figure 1A).[32,33] Therefore, balancing the sensitivity and assay time poses a major challenge in the development of future POC diagnostics (PCR-level sensitivity within 30 min), as indicated in Figure 1A. The specificity of LFAs is also important and is mainly improved through assay optimization and the identification and use of high-affinity and highly specific reagents. Figure 2 provides an overview of a sandwich LFA structure and the sample enrichment, assay optimization, and signal amplification methods for enhancing the sensitivity and specificity of LFAs.

In the following sections, we discuss strategies for improving the sensitivity (Improving Sensitivity) and specificity (Improving Specificity) of LFAs in detail. We also provide perspectives on the further improvements needed to extend the existing technologies into future POC diagnostics in each subsection.

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

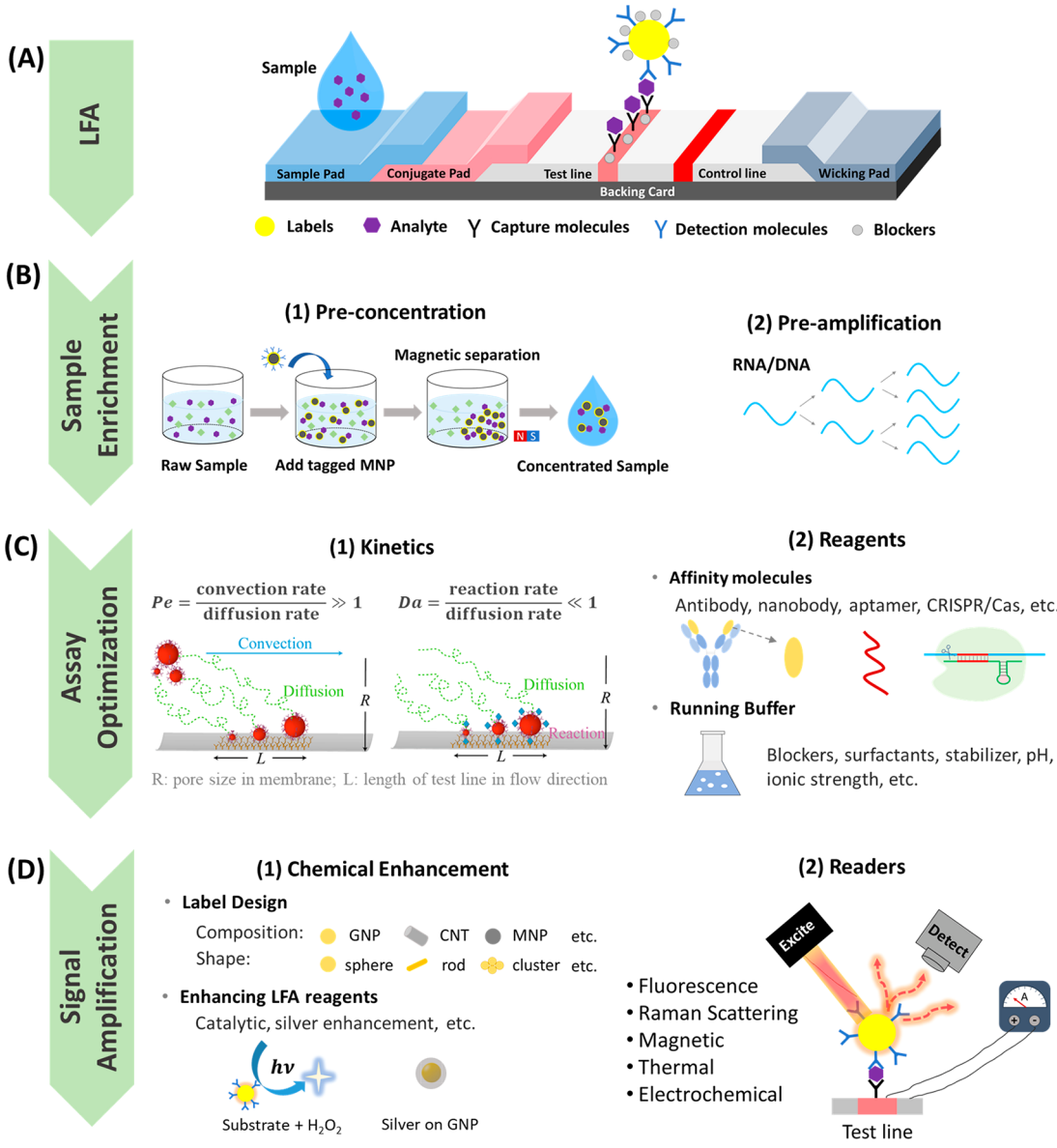


Figure 2. Overview of strategies to improve the sensitivity and specificity of lateral flow assays (LFAs). (A) Schematic of a sandwich LFA. (B) Sample enrichment: The analyte in a sample can be preconcentrated and/or amplified to enhance the detection limit. (C) Assay optimization: Assay performance can be improved by altering the assay kinetics and reagents. (D) Signal amplification: Signals from LFAs can be directly enhanced by either chemical enhancement methods or the use of readers. Related nomenclature is summarized in Table S1. MNP: magnetic nanoparticle; GNP: gold nanoparticle; CNT: carbon nanotube. Figure C(1) was reprinted with permission from ref 49. Copyright 2017 American Chemical Society.

Note that the bulk of the work reviewed here is based on prototyped laboratory LFAs from published data instead of commercial LFAs. We also note that this Perspective focuses mainly on protein- and nucleic-acid-based sandwich LFAs (see the structure in Figure 2A). However, the principles and techniques discussed here are not limited to biomolecule detection and can be extended to other targets, such as extracellular vesicles,[34−36] bacteria,[37,38] viruses,[39] fungi,[40] foodborne pathogens and toxins,[41] and toxic pollutants.[42]

IMPROVING SENSITIVITY BY ASSAY IMPROVEMENT

The sensitivity of LFAs can be substantially improved *via* either assay improvement or sample enrichment. Assay improvement includes the optimization of the fundamental assay kinetics (Figure 2C(1)) and signal amplification methods by either chemical enhancement and reader use (Figure 2D). The perspectives on their future improvements was also discussed.

Optimization of the Assay Kinetics. Exploring the assay kinetics (*i.e.*, the transport and reaction kinetics) is a fundamental step in LFA development and is essential for enhancing the LFA sensitivity (see Figure 2C(1)). These kinetics ultimately impact the specific binding (SB) and nonspecific binding (NSB) events, which, in turn, determine the sensitivity and specificity of the assay.[49−51] Therefore, the goals of optimizing the assay kinetics are to (a) maximize SB and (b) minimize NSB. Quantitative assay optimization can be achieved by maximizing the signal-to-noise ratio (SB/NSB).[51] In this subsection, we first introduce the fundamentals of assay

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

kinetics and then discuss methods to increase SB and to minimize NSB. Related work is tabulated in Table S3.

The kinetics of transport and reaction are characterized by the Péclet number (Pe) and the Damköhler number (Da), respectively, which are defined as

$$Pe = \frac{\text{convection rate}}{\text{diffusion rate}} = \frac{UR}{D} \quad (1)$$

$$Da = \frac{\text{reaction rate}}{\text{diffusion rate}} = \frac{C_R k'_{on} R}{D} \quad (2)$$

where U is the velocity of the moving fluids through the LFA membrane, R is the characteristic size of the pores inside the membrane, D is the diffusivity of the molecules and conjugation labels transported in the fluids, and C_R is the concentration of capture molecules in the test region.[49] The term k'_{on} is the effective forward immunoreaction rate constant for the conjugated label in the test region and is assumed to be $k'_{on} = nk_{on}$, where k_{on} is the forward reaction rate constant of the single antibody−antigen immunoreaction and n is the effective number of antigens per label particle.[49,52]

According to the Pe and Da estimations in Table 1, LFAs are limited by the reaction rate,[49,52] and, thus, improving the reaction efficiency is the most critical step toward maximizing SB and boosting the sensitivity of LFAs. As shown in Table 1, the transport of molecules and labels is limited by the diffusion rate (≪ convection rate, Pe = $10-10^2$), and the surface reaction is limited by the reaction rate (≪ diffusion rate, Da = $10^{-4}-10^{-1}$). This improvement in reaction efficiency can be achieved by increasing the reaction rate, which is proportional to the reaction rate constant (reaction kinetics) and reactant concentration and/or by increasing the reaction time.

Table 1. Estimated Values of *Pe* and *Da* in LFAs

parameters		estimated range of values	ref
characteristic pore size of membrane	L (m)	$(3-20) \times 10^{-6}$	53, 54
average fluid flow velocity	U (ms^{-1})	$(0.5-3) \times 10^{-4}$	49, 55
diffusivity (antigen, particles)	D (m^2 s^{-1})	$10^{-12}-10^{-10}$	49, 55
association rate constant	k'_{on}(M^{-1} s^{-1})[a]	10^3-10^5	49, 52, 56−59
concentration of capture antibodies	C_R (mol m^{-2})	$10^{-10}-10^{-8}$	49
Péclet number	$Pe = \frac{UL}{D}$	$10-10^2$ (≫1 diffusion limit)	49, 52
Damköhler number	$Da = \frac{C_R k'_{on} L}{D}$	$10^{-4}-10^{-1}$ (≪1 reaction limit)	49, 52

[a] 1 M^{-1} s^{-1} = 10^{-3} mol m^{-3} s^{-1}.

Boosting the Reaction Rate by Increasing the Reaction Kinetics. There are several reports on increasing the reaction kinetics associated with forming the conjugation/antigen/capture antibody ternary (sandwich ternary). Liang *et al.* found that the sandwich ternary forms more slowly when an antigen first binds with a conjugated label and then a capture antibody in a premixing flow than when it first binds with a capture antibody and then a conjugated label in a sequential flow.[60] As a result, the limit of detection (LoD) for malarial protein from a sequential flow was reported to be 4- to 10-fold lower than that obtained from a premixing flow.[60] Note that longer assay times can be a drawback of sequential flow. For nucleic acid hybridization, the hybridization kinetics are strongly correlated to the ionic strength at a salt concentration below 0.2 M.[61] Thus, He *et al.* added a saline barrier in the membrane before the test region to accelerate the hybridization reaction, although this addition also slowed the flow velocity and increased the assay time.[62] As a result, they achieved a 10-fold increase in detection sensitivity without changing the LFA format and procedures.[62] The above-mentioned work is summarized in Table S3.

Boosting the Reaction Rate by Increasing the Reactant Concentration. For most immunoreactions between antigens and antibodies, the reaction rate constant is relatively unchangeable. Thus, concentrating the reactants can be an effective alternative way to increase the reaction rate and, subsequently, to increase the number of captured labels in the test region. First, analytes in a sample can be preconcentrated before the sample is introduced to an LFA test (see Figure 2B(1)). For example, Sharma *et al.* used magnetic separation together to preconcentrate analytes in a sample prior to lateral flow and achieved a 10-fold increase in sensitivity.[63] Mashayekhi *et al.* reported that analytes predetected by labels can also be preconcentrated in a micelle-poor layer by adding Triton X-114, a nonionic surfactant, into the sample to form a two-phase micellar system.[64] This method was reported to provide an *ca.* 10-fold decrease in the LoD.[64]

Second, analytes can also be concentrated during the flow period of an LFA. For instance, isotachophoresis (concentrating flowing ionic analytes with an applied electrical field) helps to preconcentrate the antigen−conjugation complex and to enhance the transport kinetics in LFAs. As a result, the surface reaction rate and equilibrium binding of labels are dramatically increased in the test region.[56] Experimental results show that this technique improves the LoD by up to 400-fold.[56]

Third, increasing the number of effective binding sites for the conjugated labels can augment the reaction rate and thus lower the LoD. This increase in binding sites can be realized through either label design or the specific orientation of detection molecules. For example, the size of the gold nanoparticle (GNP) labels can be increased,[49] or the particle surface can be functionalized with multiple layers[65] to enable the loading of more detection molecules, resulting in increased numbers of binding sites. Of note, the particle size and coating are limited by the need to maintain the particles' stability and diffusivity in flow through the porous membrane; otherwise, staining or background noise due to settling out and/or NSB may occur.[49] Enforcing a specific orientation of the detection molecules on labels' surface through improved conjugation methods was reported to generate more effective binding sites than were achieved with randomly oriented conjugation through traditional physical adsorption.[66−68] A specific orientation can be achieved through either covalent binding mediated by a chemical layer (*e.g.*, PEGylation) or bioaffinity binding mediated by a biomolecular layer (*e.g.*, protein A and G, biotin−streptavidin coupling, DNA-directed immobilization).[66−68] Furthermore, the coverage of the detection molecules should be optimized to minimize any steric hindrance created by a dense layer of detection molecules and to maximize the affinity for the analyte.[69]

Fourth, the number of effective binding sites in the test region can be increased. For instance, regular capture molecules at the test line can be substituted with three-dimensional (3D) "proteinticle" probes in which multiple peptides are self-assembled and oriented to give a substantial

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

enhancement in reactivity, leading to a 4- to 8-fold improvement in sensitivity.[70] Modifying the membrane with cellulose nanofibers to enable the loading of more capture molecules can also boost the detection sensitivity by 20-fold.[71] Cellulose nanofibers were also reported to bring the capture molecules closer to the surface, thus increasing the colorimetric intensity of the captured labels by 36.5%.[72]

Increasing the Reaction Time. Increasing the reaction time can also augment the number of captured labels in the test region. For example, putting cotton threads into the membrane can slow the flow rate and improve the detection sensitivity by 4-fold.[73] Adding a stacking pad between the membrane and the conjugation pad can also lengthen the reaction time, leading to 1.1- to 2-fold decreases in the LoD.[74]

In addition to maximizing SB by the aforementioned methods, minimizing NSB is also important to improve the sensitivity of LFAs because NSB can interfere with the detection of very low concentration analytes and pose limits to sensitivity improvements. Thus, reducing NSB in combination with performing other assay optimization methods can improve the LFA sensitivity. For example, the PEGylation of 40 nm gold nanospheres (GNSs) led to a large decrease in NSB, which otherwise arises from the aggregation of unstable citrate-stabilized GNSs.[75] As a result, the detection sensitivity for bisphenol A increased by 12.5-fold.[75] Likewise, introducing a silica coating on GNPs significantly lowered the background noise by imparting high particle stability.[76] This coating also increased the surface area, which enabled the loading of more antibodies.[76] Subsequently, the LoD for alpha-fetoprotein by silica-coated GNPs was lowered by 30-fold.[76] In another example, coating GNPs with polydopamine increased the particles' tolerance to pH and ionic strength, thereby reducing possible NSBs.[77] This coating also augmented the antibody loading efficiency. As a result, the sensitivity of the label-optimized LFAs was 10-fold higher than that achieved with traditional, bare GNP labels.[77] A summary of related work is provided in Table S3.

In summary, optimizing the assay kinetics is a fundamental step toward improving LFAs that can achieve up to hundred(s)-fold increase in sensitivity (see Table S3 and Figure S1). Even greater sensitivity improvements can then be achieved by amplifying the signals from test regions (see Tables S2 and S3 and Figure S1), as discussed in the following two subsections.

Chemical Enhancement. In addition to optimization of the assay kinetics, sensitivity can also be extensively improved by amplifying the signal from the test region. Increasing the colorimetric contrast of the positive test region through chemical enhancement is a direct and straightforward way to amplify the signal while maintaining the convenience of visual detection. This enhanced contrast can be realized through label design and the use of enhancing LFA solutions (see Figure 2 D(1)). Related work is summarized in Table S3.

Label Design. Label design usually replaces the traditional small (*ca.* 20−40 nm) GNSs employed in LFAs with other labels that have stronger colorimetric contrast while maintaining the traditional LFA format. Stronger contrast can be achieved by modifying the structure and size of the GNPs or by replacing the GNPs with particle clusters or particles made of another metal, metal oxide, or organic material, as shown in Figure 2 D(1). For example, a label of GNP-decorated silica nanorods (*i.e.*, microsized silica nanorods coated with colloidal gold), achieved a *ca.* 50-fold lower LoD in the detection of rabbit IgG than traditional GNSs.[78] Similarly, polystyrene microbeads have been used as a support for nm-scale GNSs to enhance the colorimetric contrast of the test region; this design improved the detection sensitivity for the influenza virus H3 subtype by 64-fold over that achieved with 10 nm GNP-based LFAs and 16-fold over that achieved with 30 nm GNP-based LFAs.[79] In another study, the use of gold nanopopcorn achieved a 5-fold lower LoD for procalcitonin relative to that obtained from LFAs that used 20 nm GNPs.[80] Strikingly, the use of carbon nanotubes (CNTs) as a label in the detection of rabbit IgG decreased the detection limit by 3 orders of magnitude relative to the use of the traditional GNP-based LFAs. This improvement arises from the higher aspect ratio of CNTs, which enables the loading of more detection antibodies and thereby improves the immunoreaction rate.[81] The label design approach preserves the LFA benefits of a rapid response, simple use, and low cost without changing any assay formats or steps.

Use of Enhancing Lateral Flow Assay Reagents. Enhancing LFA reagents can be applied to induce catalytic or other chemical reactions in the test region after a normal assay to amplify the colorimetric contrast. Catalytic amplification is usually achieved by using an enzyme or nanozyme to catalyze oxidation−reduction reactions in the test region. The most widely used enzyme is horseradish peroxidase (HRP), which catalyzes the oxidation of an organic substrate in the presence of hydrogen peroxide. In LFAs, HRP is linked to detection molecules that are conjugated with labels to be captured in the test region. After a sample is run on the LFA, a wash step is performed to remove excess labels from the membrane. Finally, solutions of the HRP substrate and H_2O_2 are flowed through the LFA to achieve enzymatic amplification and produce a strong color enhancement or chemiluminescence in the test region.[82] Parolo *et al.* reported an increase in sensitivity of up to 1 order of magnitude over conventional GNP-based LFAs in the detection of human IgG by applying enzymatic amplification.[83]

Recently, nanozymes (*i.e.*, nanomaterial-based artificial enzymes) have been discovered and rapidly developed as direct surrogates of natural enzymes (*i.e.*, protein-based) for catalysis. Compared with natural enzymes, nanozymes have advantages of higher catalytic stability, easier modification process, and lower manufacturing cost, among others (see ref 84 for a comprehensive review of the design of nanozymes, their applications in diagnosis and therapeutics, and the outlook of this technology).[84] Some nanozymes have already been used as labels in LFAs. For example, a Pt nanocatalyst (Au@Pt core@shell structure) achieved an LoD for p24 spiked in sera as low as *ca.* 0.8 pg/mL (*ca.* 33 fM), which is even better than the sensitivity of commercial ELISA (>1 pg/mL, >42 fM).[85] Similarly, other LFAs have used Au@Pt core@shell nanostructures, such as Pt-decorated GNPs and Pt−Au nanoflowers, for catalytic amplification and achieved LoDs that are approximately 100-fold lower than those of conventional GNP-based LFAs.[86,87]

Other chemical enhancement techniques include silver enhancement, double gold conjugation, and induced gold aggregation. In the silver enhancement method, Ag is nucleated on captured GNPs in the test region by flowing Ag-reducing reagents through an LFA after a normal assay. The resulting Ag layer on the GNP label surface amplifies the color intensity of the test region. With this method, a sensitivity gain of approximately 10-fold relative to that

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

without Ag enhancement has been achieved.[80,88] Similarly, for double gold conjugation, secondary GNPs are introduced to bind with the primary GNPs that are already captured within the test region, which results in an enhanced color intensity. This binding can be achieved by making use of the high biotin−streptavidin binding affinity[89] or by employing the reaction between primary and secondary antibodies, which is similar to the basis of indirect ELISA.[90] The double gold conjugation method has been reported to increase the detection sensitivity for hepatitis B surface antigen by approximately 30-fold.[89] The induced gold aggregation method is similar to the double gold conjugation approach, but more GNPs can be coated on the captured GNPs with this method, thus better amplifying the color intensity. For example, the direct use of aggregated MNPs as labels was found to improve the detection sensitivity by 40-fold over that achieved with monodisperse MNPs, although large aggregates have a slower transport velocity and thus require a longer assay time.[91] Alternatively, using a liposome-encapsulating reagent to aggregate additional GNPs onto the captured GNPs in the test region achieved a 1000-fold improvement in sensitivity.[92] More impressively, adopting DNA−GNPs to induce the 3D growth of GNP aggregates led to a decrease in the LoD by 4 orders of magnitude relative to that without signal amplification.[93] These enhancements are also noted in Table S3. Importantly, although these approaches increase the sensitivity, they come with a reduction in the ability to quantify the signal (*i.e.*, the originally captured labels) due to the inconsistency associated with coupling multiple reaction steps.

Reader Use. In addition to the chemical enhancement method, LFA signals can also be amplified with the use of readers. With readers, captured nanoparticle (NP) labels in the test region are excited by an external physical stimulus, such as laser light, an electric potential, or a magnetic field (see Figure 2D(2)) to produce an amplified signal. The amplified signal is then detected by sensitive optical/electrical/magnetic sensors that can discern tiny signal differences over the background. By applying intense external fields and sensitive sensors, reader systems can enhance the detection sensitivity by several orders of magnitude over the traditional visual readout and rival the sensitivity of laboratory-based ELISA (see comparison in Figure 1A). Additionally, quantification of a signal (*i.e.*, the amount of label) can be achieved with reader systems because the signal intensity is generally proportional to the number of NPs captured in the test region, which also correlates with the amount of target analytes. Multiple types of readers are available depending on the excitation method, including fluorescence,[42,94] surface-enhanced Raman scattering (SERS),[95,96] photothermal (*i.e.*, thermal contrast,[43,44] photoacoustic imaging,[45,46] photothermal laser speckle imaging,[47] and thermal photonic lock-in imaging[48]), electrochemistry,[97] and magnetic amplification.[98,99]

Due to the significant benefits associated with reader use, these systems have attracted substantial attention and are being applied in next-generation LFA technologies, as described in multiple reviews.[11−15,32,100,101] Nguyen *et al.* reviewed the majority of these reader systems and discussed their working mechanism, setup, development, and detection sensitivity.[13] Kim *et al.* summarized various NPs and their roles in different reader-assisted LFAs and compared their detection sensitivity to those of commercial tools such as traditional commercial LFAs, ELISA, and PCR.[100] Ye *et al.* extensively reviewed signal amplification methods based on the laser excitation of plasmonic NPs.[15] In addition to the working mechanism and the advancements of the reader systems, the authors discussed how to bridge the gap between laboratory readers and mature commercial needs.[15] In addition to standard electronic readers, smartphone-based readers are also emerging as a promising POC technology, and additional details are discussed in other reviews.[12,102−104]

Future of Assay Improvement. In this section, we provide a comprehensive comparison of the LFA techniques discussed above and offer perspectives on the likelihood of those techniques becoming mature products or meeting future POC diagnostic needs. The sensitivities and detection times of the techniques are systematically compared with those of the existing commercial tests (commercial LFAs, ELISA, and PCR) in Figure 1A. A detailed comparison of the various signal amplification methods (labels, reagents, and readers) is presented in Figure 1B with the corresponding literature summarized in Table S2. Their advantages, disadvantages, and potential for application in future ultrasensitive (sub-fM), rapid, easy-to-use, low-cost, and multiplexed POC diagnostics are discussed.

The sensitivity comparison shows that a substantial improvement was achieved with these advanced LFAs, but there remains a need for integrated assay optimization to reduce performance variations within the same technique. Figure 1A shows that the sensitivity of novel LFAs is 1−9 orders of magnitude better than that of traditional commercial LFAs and that it overlaps with the sensitivity of ELISA. The sensitivity of some of the techniques, such as fluorescence LFA[105] and electrochemical paper-based diagnostics,[106] can even reach the fM level (the lower limit of the ELISA sensitivity). However, Figure 1B also indicates that there is a large variation in the LoD (3−6 orders of magnitude) within the same technique. This variation results from insufficient optimization of the assay. During an assay, NSB of the labels inevitably occurs along with SB in the test region. When signal amplification is performed, both the signal (from SB) and the noise (from NSB) are amplified; thus, the number of FNs is reduced, but the number of FPs may also increase. In other words, the sensitivity improves at the cost of the specificity. In particular, for low target concentrations, the amount of label captured through NSB can be comparable to or even larger than that captured by SB. In that case, further increasing the intensity of the external excitation field cannot discriminate the signal from the noise, and instead, it creates FPs. This issue has been reported for LFAs that use thermal contrast amplification[51] as well as silver enhancement.[107] As a result, the efficacy of signal amplification can be limited by the NSB of labels.

To address this limitation, an integrated assay optimization method that is compatible with the signal amplification technique is needed (see flowchart in Figure 3). This optimization method is different from the traditional LFA development protocol,[108,109] because the latter does not include signal amplification in the iterative assay optimization process. In contrast, in this integrated assay optimization approach, an assay optimization step is performed after signal amplification specifically to reduce NSB. After such optimization, a more intense applied field or enhancing LFA reagents (*e.g.*, silver enhancement) can be used to enhance the detection sensitivity further until the specificity suffers (*i.e.*, the number of FPs increases). This iterative assay optimization and signal amplification approach can be continued until the sensitivity

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

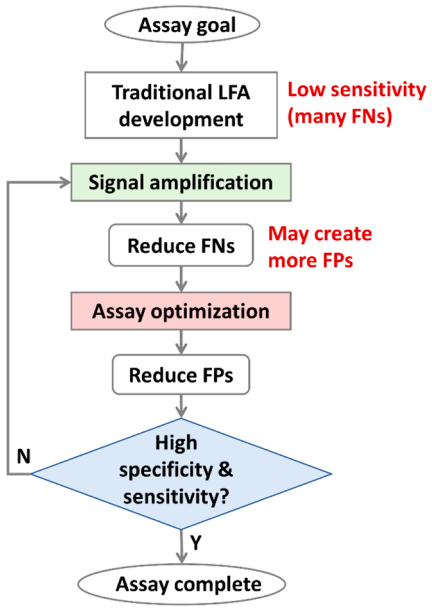


Figure 3. Integrated assay optimization with signal amplification methods. The traditional lateral flow assay (LFA) development steps are described in other work.[108,109] FPs: false positives; FNs: false negatives.

and specificity are sufficiently high. To achieve quantitative assay optimization, Zhan *et al.* showed how the signal-to-noise ratio (*i.e.*, SB/NSB ratio) can be maximized.[51] This ratio is related to the signal *versus* noise in the test region when the assay is run in the presence and absence of the target analytes. Alternatively, the control lines can be precoated with target molecules,[51] thus giving SB-dependent signals. The SB/NSB ratio can be quantified by an imaging tool or by readers. To achieve an even higher sensitivity, multiple sensitivity-enhancing methods can be combined in the same LFA, although integrated assay optimization is still needed.

In addition to sensitivity, speed, ease of use, and cost are important metrics in the evaluation of techniques for application in POC diagnostics. A summary of the advantages, disadvantages, and necessary future improvements of LFAs is provided in Table 2 (with specific innovations in Tables S2 and S3), and a detailed comparison of their detection times is shown in Figure 1B. As noted in Table 2, chemical enhancement maintains the advantage of rapid visual detection. More specifically, label design usually does not impact the assay time, although the diffusion issues of large labels and particle-dependent NSB should be considered. In contrast, the use of enhancing LFA reagents can significantly add to the assay time, assay complexity, and imprecision because additional steps are needed to deliver the various reagents. The assay time can then range from near 20 min[85] to approximately 1 h.[110,111] To address this increase in assay time, some researchers have sought to automate the multiple solution-delivery steps by designing novel LFA structures. For example, a two-dimensional paper network with internally set timings was designed to automate a multistep assay,[111] but its assay time was still long (*ca.* 1 h).[111] Another upgraded device took advantage of a polymer that swelled upon the addition of water to automate the sequential delivery of the immunoreaction and amplification reagents. This approach achieved assay completion within 20 min.[112] Further improvements can be made by simultaneously miniaturizing the device, automating all assay steps, and reducing the assay time by exploring the reaction kinetics.

Similarly, external signal amplification with readers can also add to the detection time and cost of an assay, although this method has the additional advantage of providing reproducible quantitative readouts. The increase in detection time introduced by most readers is usually <20 min except for the electrochemical measurement. The total time consumed by the electrochemical method is usually <30 min,[106,113,114] but some assays with multiple steps can take over 1 h.[115] Many efforts have been made to reduce the scanning time of these readers. For example, the fastest SERS reader can scan a test region in only 5 s,[96] and a thermal contrast reader with a scanning time of <1 min is being developed.[116] Some of the efforts to reduce scanning time are extensively discussed in a separate review.[15] The reliability and reproducibility of the reader performance in various POC conditions also remain unknown. Therefore, further work on miniaturization, cost reduction, and validation through clinical trials is needed to make these readers better suited for POC applications.

In addition, there is a strong drive to develop multiplexed LFAs that are capable of simultaneously detecting multiple analytes due to the associated advantages of a reduced cost,

Table 2. Advantages, Disadvantages, and Necessary Future Improvements of Lateral Flow Assays

signal amplification	advantages	disadvantages	future improvements
chemical enhancement	sensitivity increase fast visual readout	NSB[51] diffusion issues with large labels[49] adds time and complexity[110,111] may not be quantified[76]	miniaturization automation simplification integrated assay optimization (SB/NSB) multiple analyte detection combination approaches novel signal processing methods
readers	sensitivity increase quantification[13,15,100] reproducibility[13,15,100]	NSB[51] adds steps and time[13,15,100] adds cost[13,15,100] complex structure (electrochemical)[114,121]	clinical validation miniaturization cost reduction integrated assay optimization (SB/NSB) multiple analyte detection combination approaches novel signal-processing methods

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

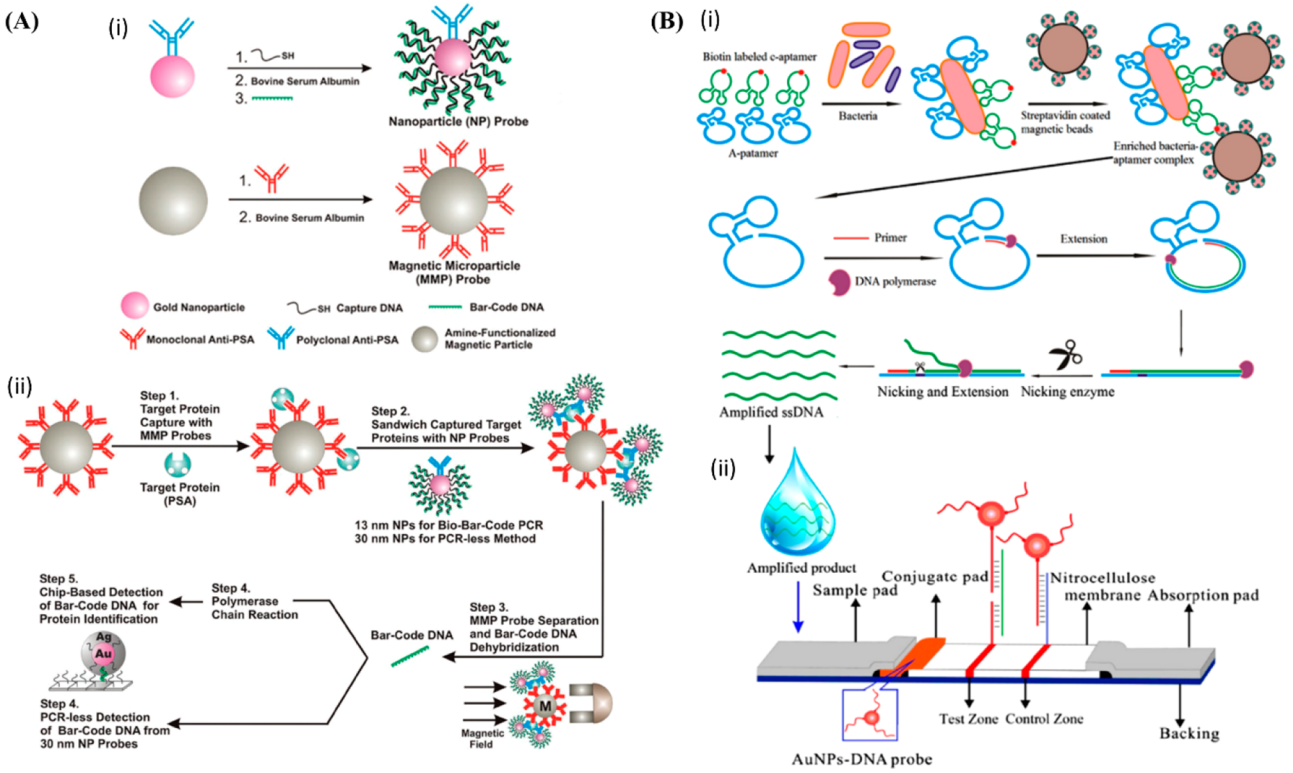


Figure 4. Indirect sample preamplification for the polymerase chain reaction level detection of proteins and bacteria. (A) Use of nanoparticle-based biobarcodes and magnetic microparticles for the ultrasensitive detection of proteins.[135] (B) Ultrasensitive and DNA extraction-free detection of salmonella based on aptamer-mediated strand displacement and amplification.[137] (A) Reprinted with permission from ref 135. Copyright 2003 AAAS. (B) Reprinted with permission from ref 137. Copyright 2014 Elsevier.

lower sample volume, and the ability to discriminate rapidly between diseases with common symptoms. However, several challenges with multiplexed LFAs remain, including cross-reactivity between affinity molecules for different target analytes, physical limitations of multiplexed test regions in an LFA strip, and difficulty in clinical validation, which requires patients with multiple co-infections.[117] More in-depth discussion on the development, challenges, and opportunities of multiplexed LFAs can be found in other work and reviews.[12,103,117−119] Note that vertical flow assays (VFAs), which operate under similar fundamental principles as LFAs but have a different flow direction, can increase the multiplexing capability and reduce cross-reaction effects.[97,120] A detailed comparison of LFAs and VFAs is available in other reviews.[103,104]

Finally, novel signal-processing methods are emerging to enable sub-fM detection sensitivity. The signals from LFAs are usually read in an analog format, which intrinsically limits the upper limit of sensitivity improvements, that is, the readers require millions of labels to be accumulated to generate a detectable signal intensity. As a result, it is difficult for these techniques to achieve sub-fM sensitivities similar to that of ELISA, as shown in Figure 1A. In contrast, digital ELISA (dELISA) enables the detection of sub-fM to aM concentrations (*i.e.*, PCR-level sensitivity or single-molecule detection in 1 μL of solution) owing to its digitized signal acquisition method.[122,123] dELISA can count the signal from each enzyme label after immunoreaction.[122,123] The state-of-the-art commercial dELISA system is the signal-molecule array (Simoa) and the corresponding Simoa HD-1 Analyzer.[122,124] The Simoa enables partitioning of hundreds to thousands of paramagnetic beads into femtoliter wells. Each well can hold at most one bead, thus concentrating the immunoreaction product from a single target molecule in a femtoliter droplet.[122,124] By counting the signals from each well, dELISA can achieve a *ca.* 1000-fold improvement in sensitivity over conventional ELISA.[122,124] However, the equipment costs *ca.* 100,000 USD, and a testing operation takes *ca.* 1 h to complete, although multiple samples can be loaded together.[124,125] To lower the cost and detection time, many efforts have focused on upgrading the Simoa platform; these efforts include droplet dELISA,[126] droplet-free dELISA,[127] mobile dELISA,[125] and dropcast single-molecule arrays.[128] Of note, these platforms are not yet ready for POC use because of either the complexity of their fabrication and use or the long turnaround times required for signal processing. Nevertheless, it is valuable to consider combining the signal acquisition method of dELISA with existing LFAs to develop a cheaper and simpler (sample-to-answer) digital detection platform for ultrasensitive (sub-fM or aM) POC diagnosis. This goal may be achieved by taking advantage of porous membranes that allow automated fluid flow, reagent storage, mixing, and volumetric reactions and transparent plastic containers that enable imaging-assisted digital signal acquisition.

Miller *et al.* proposed another signal processing method for spin-enhanced LFAs to enable sub-fM detection of labels.[129] The authors modulated the fluorescence intensity of nanodiamond labels by a microwave field (spin manipulation), hence the fluorescence from labels could be separated from background autofluorescence in the frequency domain with lock-in analysis. As a result, this method achieved a 10^5-fold increase in sensitivity for the avidin−biotin (direct binding)

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

assay than traditional GNP-based LFAs.[129] Note that in sandwich LFAs, NSB of nanodiamond labels in the test region can pose a limit to the sensitivity improvement. Thus, integrated assay optimization (Figure 3) will still be needed. Overall, after assay optimization, device upgrade, and validation, this method holds promise to be adapted for future ultrasensitive POC use.

IMPROVING SENSITIVITY BY SAMPLE PREAMPLIFICATION

In addition to improving LFAs, preamplifying the analyte (Figure 2 B(2)) is also an effective way to boost sensitivity. Both nucleic acids (direct amplification) and other analytes (indirect amplification) can be amplified prior to LFA testing, leading to PCR-level (aM) sensitivity.

Amplifying Nucleic Acid Analytes. The gold standard and most widely used sample preamplification tool is PCR, which can achieve aM detection sensitivities for target DNA/RNA (see Figure 1A).[100] However, PCR cannot be used in POC settings due to the need for specialized equipment, trained operators, and a strictly controlled working environment.[6] To address this limitation, isothermal amplification methods, which can amplify nucleic acids at a constant temperature without the need for thermal cycling, have been proposed as a substitute for PCR with the goal of decentralizing ultrasensitive DNA/RNA diagnosis from the laboratory to POC settings. Multiple isothermal amplification methods are under development, and some are capable of completing the preamplification step within 30 min, as featured in many recent reviews,[130−132] and are being commercialized.[132−134] The readout format of these methods can be either real-time or end point. The real-time readout is usually based on fluorescence and needs an excitation light source and a photodetector, which is not ideal for POC tests due to the added cost and energy consumption of this equipment. In contrast, LFAs can utilize a simple end point readout. In summary, as shown in Figure 1A, analyte amplification with an LFA can yield PCR-level results at the cost of a somewhat greater complexity, potentially reduced dynamic range, and longer time duration (>30 min) compared to typical LFAs.

Amplifying Analyte-Specific Aptamers. Unlike DNA and RNA, other targets such as proteins cannot be directly amplified and therefore are difficult, if not impossible, to achieve PCR-level detection by LFAs. However, novel methods for amplifying target-related DNA or target-specific aptamers have been proposed, as shown in Figure 4. Figure 4A presents the biobarcode amplification method proposed by the Mirkin group.[135] In this assay, target proteins are dually recognized by antibody-conjugated GNPs and magnetic microparticles. The GNPs are also pre-encoded with biobarcode DNA in addition to detection antibodies. After magnetic separation, the biobarcode DNA fragments are released from the NPs, after which they are amplified and detected. The detection of the biobarcode DNA indicates the presence of the target proteins. This method lowered the detection limit for the protein of interest to as low as 30 aM (within PCR-level sensitivity). A similar method was also applied to the detection of target DNA with an LoD of 0.5 aM, which is also a PCR-level sensitivity.[136] Figure 4B shows another example of indirect amplification in which aptamers are developed to specifically recognize a target and become amplified after magnetic separation.[137] In this method, target bacteria are first recognized by two aptamers. One of the aptamers is linked to magnetic beads to enable magnetic separation, while the other is amplified and detected afterward. This method achieved a detection limit for *Salmonella enteritidis* of as low as 10 colony forming units without DNA extraction.[137] However, these assays require multiple steps and take hours to complete. Furthermore, the extensive manual manipulation required in this technique may compromise the reproducibility. Therefore, further automation and reduction of the assay time are needed to make this technique suitable for future large-scale POC use.

The Future of Sample Preamplification. Although the amplification of targets or target-specific aptamers provides access to PCR-level detection sensitivities, challenges remain in further advancing these technologies to achieve future ultrasensitive and highly specific POC testing, which requires that they be simple (sample-to-answer), rapid (<30 min), and inexpensive.

First, further investigation is needed to develop robust "sample-to-answer" devices that can integrate all steps, including DNA/RNA extraction, amplification, and end point readout. The inclusion of multiple steps increases the assay complexity, time, and labor and contributes to a lack of robustness and inaccuracy. To address these drawbacks, microfluidic platforms for automating these assay steps are in development.[134,138] However, these platforms usually require electrical pumps or centrifuges.[134,138] To eliminate the need for electrical equipment, microfluidic platforms such as 3D-structured microfluidic paper,[139,140] capillary tubes,[141] and vacuum-powered microfluidic chips[142] have been developed. Although these are intriguing developments, these assays require careful further development and validation to ensure that they are robust.

Second, the added sample preamplification step increases the total detection time. As shown in Figure 1A, most systems that combine isothermal amplification with fast readout still take 1/2−4 h to complete. The reaction itself has been studied in depth to improve the kinetics of the amplification step. For example, among the various isothermal methods, recombinase polymerase amplification (RPA),[143] loop-mediated isothermal amplification (LAMP),[144,145] and exponential amplification reaction (EXPAR)[146,147] can complete the amplification reaction within 1 h. Further, some real-time RPA detection methods can be completed within 10−20 min.[148−150] More examples are summarized in a recent review article.[143] Note that the sensitivity may be compromised by cutting down the preamplification time and, thus, combining sample preamplification with an existing assay improvement method for the end point LFA readout may be a promising avenue.

Third, nonspecific sample preamplification can occur, which makes the assay less specific. This issue has been reported with isothermal methods[134,151−153] such as LAMP,[154] RPA,[151,152] rolling circle amplification (RCA),[134] helicase-dependent amplification (HDA),[134] and EXPAR.[153] To minimize nonspecific sample preamplification, it is critical to optimize the primer design and assay conditions.[133,151] Another promising way to maintain specificity is with the newly discovered clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems.[155,156] The CRISPR/Cas systems are highly specific to target sequences, thus enabling highly specific detection.[157] However, challenges with incorporating the CRISPR/Cas reaction into future "sample-to-answer" POC devices remain.

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

Fourth, the dynamic detection range of analytes may be reduced by sample preamplification due to saturation in the exponential amplification step.[158] This reduction can be problematic because the quantification of analytes in a wide concentration range is important to clinical treatment. To address this limitation, the amount of analyte and primers in the amplification step needs to be optimized.

Finally, other intrinsic issues related to sample preamplification also need to be addressed. These issues include the tolerance to impurities and inhibitors, the presence of amplification bias, and the occurrence of cross reactions in multiplexed amplification. More details about and insights into these issues can be found in other reviews.[133,159] Above all, sample treatment (purification), device innovation (reagent mixing to enhance the reaction), and reagent optimization and screening (for multiplexed sample preamplification and detection) will need to be further investigated to make assays robust for POC use.

To summarize the methods for improving sensitivity, advanced assay improvement techniques (assay kinetics optimization, chemical enhancement, and reader use) enable LFAs to achieve ELISA-level (pM−fM level) sensitivities, although further effort is needed to bring them to successful commercialization for POC use. The combination of sample preamplification with an LFA readout also holds promise for future rapid (<30 min) and ultrasensitive (PCR-level or aM-level) POC diagnostics after the challenges mentioned above are addressed.

IMPROVING SPECIFICITY

Specificity is equally important as sensitivity in the development of a robust POC diagnostic test. Improving the specificity relies on not only minimizing the NSB but also screening for the most specific affinity molecules.

Assay Optimization. Assay optimization to reduce the NSB that results in an FP readout is critical to improving the specificity of LFAs. The NSB of labels in the test region usually arises from the following sources:

- Presence of NSB substances in a sample that are capable of binding to the affinity molecules in LFAs[160]
- Nonspecific interactions between the conjugated labels and the capture antibodies and/or membrane[51,75,161]
- Physical trapping of the conjugated labels (especially aggregates of unstable labels) in the porous membrane[49,51,75]

To address these issues, the following approaches are outlined in the sections below.

Reducing Substances Capable of Nonspecific Binding. Multiple methods are available for treating samples to reduce substances capable of NSB, as summarized in Table 3. For example, when detecting analytes from whole blood, blood cells and other large particles are usually separated from the blood before the LFA is run by adding a filtration pad before the conjugation pad[162] or by centrifuging the sample. Also, preheating urine was found to reduce the activity of thermally labile biomolecules, thus decreasing the number of FPs obtained when detecting cryptococcal antigen by LFAs.[163]

For DNA/RNA LFAs with a sample preamplification step, FPs can be produced by the primers/probes and side products from the preamplification step, such as primer dimers.[160,164,165] Such FPs can be reduced by optimizing the primer and probe sequences in the preamplification step[151,152,166,167] and the capture and detection sequences in the LFA.[168] Other special treatments can also be used to prevent FPs. For example, Li *et al.* used a graphene oxide pad to filter residual primers and primer dimers to increase the specificity.[160] Similarly, running LFAs at 37 °C was reported to reduce the nonspecific adsorption of nucleic acid-conjugated labels to the test region (see Table 3).[168]

Table 3. Innovations for Reducing NSB to Improve Specificity[a]

approaches	consequences	ref
Reducing Substances Capable of NSB		
blood cell pad	filter out blood cells	162
heat fresh urine	inactive biomolecules	163
graphene oxide pad	filter out primers and primer-dimers	160
primer and probe sequence	reduce side products in sample preamplification	151, 152, 166, 167
detection/capture sequence	reduce NSB to nontarget sequences	168
elevate LFA temperature	reduce NSB of conjugated GNPs at test regions	168
Other Assay Optimization Methods		
NP surface coating	stabilize NPs to avoid aggregation	75
optimal label size and concentration	reduce NSB of labels at test regions	169
blocking conjugates	reduce NSB of labels to nontarget molecules and membrane	170
blocking membrane	reduce NSB of proteins to membrane	51, 171, 172
assay running buffer screening	maximize SB and minimize NSB; preserve NP stability	51, 171, 173

[a]LFA: lateral flow assay; NP: nanoparticle, SB: specific binding.

Other Assay Optimization Methods. Other typical assay optimization methods for minimizing NSB include:

- Surface modification/blocking of labels
- Optimizing the label size and concentration[169]
- Screening the running buffer
- Membrane blocking

The work related to other assay optimization methods is summarized in Table 3 and discussed in detail below. Of note, these assay modification methods can also impact SB; thus, the signal-to-noise ratio (*i.e.*, SB/NSB ratio)[51] should also be evaluated and maximized.

First, the surface modification/blocking of labels prevents label aggregation-induced NSB by blocking NSB sites and increasing the hydrophilicity and stability of the labels. Typical blockers and stabilizers (see Table S1 for definition) include proteins (*e.g.*, bovine serum albumin (BSA)) and sugars.[170,173,174] The surface functionalization of labels, such as through PEGylation[28] and hydroxylation,[174] can also increase the particle stability and prevent nonspecific electrostatic interactions. Second, the label size and concentration simultaneously affect both SB and NSB.[49,169] Smaller labels showed lower signal intensity and sensitivity compared to larger ones, whereas labels that were too large had diffusion and NSB problems.[49,169] Likewise, insufficient label concentration led to low signal intensity, whereas too high of a concentration resulted in NSB and background noise.[168] Thus, label optimization is needed to maximize the SB/NSB ratio for the best assay performance.[51] Third, the composition of the running buffer (see Table S1 and Figure 2C(2)) has proven to be important for the overall LFA performance.[51,171] For instance, surfactants are added to the running buffer to

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

Table 4. Comparison of Affinity Molecules

type	antibody	nanobody	short peptide	aptamer
target anaytes[186]	immunogenic: proteins, haptens			any target, including ions, non-immunogenic or toxic targets, and cells
target size[186]	larger than others			smaller than antibodies
affinity[12]	high	high	high	higher
dissociation constant,[12] (M)[a]	10^{-7}–10^{-9}	10^{-6}–10^{-11}	10^{-6}–10^{-8}	10^{-9}–10^{-12}
batch difference[186]	varied	less varied		uniform
stability[186,187]	sensitive to heat, pH	more pH and heat stable than antibodies		tolerant to heat, pH, salt, and chelating agents[187]
oriented immobilization[15]	difficult, requires multiple steps	easier than antibodies		easy

[a]Note: Biotin–avidin interaction has a dissociation constant of about 1.3×10^{-15} M.[189]

Table 5. Different Cas Effectors in CRISPR/Cas Systems and Example Diagnostic Platforms[a]

types	Cas9 (dCas9)	Cas12	Cas13a
target	dsDNA	ssDNA, dsDNA	ssRNA
cleavage	target only	target and other sequences (*i.e.*, collateral cleavage)	
diagnosing platforms	RHC,[201] NASBA,[202] PC reporter,[203] CAS-EXPAR,[204] CASLFA[195]	DETCTR,[196,205] HOLMES[200,206]	SHERLOCK,[158,199,207] HUDSON + SHERLOCK[197]

[a]dCas9: catalytically dead Cas9 (inactive endonuclease); dsDNA: double-stranded DNA; ssDNA: single-stranded DNA; ssRNA: single-stranded RNA; RHC: RCA-CRISPR-split-HRP; NASBA: nucleic acid sequence-based amplification; PC reporter: Paired dCas9 PC reporter; CAS-EXPAR: CRISPR/Cas9 triggered isothermal exponential amplification reaction; CASLFA: CRISPR/Cas9-mediated lateral flow nucleic acid assay; DETCTR: DNA endonuclease-targeted CRISPR trans reporter; HOLMES: 1 h low-cost multipurpose highly efficient system; SHERLOCK: specific high-sensitivity enzymatic reporter unlocking; HUDSON: heating unextracted diagnostic samples to obliterate nucleases.

increase the hydrophilicity of biomolecules and membranes and to reduce nonspecific hydrophobic interactions; however, an excessively high concentration of surfactant adversely affects SB.[173] The pH, ionic strength, blockers, and stabilizers in the running buffer need to be tuned to preserve the stability of the labels and to reduce interference from heterophilic antibodies in patient samples while maintaining a high SB efficiency. Again, the SB/NSB ratio can serve as a quantitative metric for screening the running buffers.[51] Finally, membrane treatment prior to performing the assay is optional to reduce NSB by blocking possible NSB sites in the membrane. For example, De Puig *et al.* found that treating the membrane with human serum reduced FPs significantly.[172]

Highly Specific Affinity Molecules. Optimal affinity molecules provide maximal specificity (*i.e.*, maximal SB while minimizing NSB). The selection and screening of appropriate affinity molecules (*i.e.*, antibody or aptamer) for its analyte (*i.e.*, protein, DNA, or RNA) are critical to assay performance.[175−177] For standard single protein detection, antibody pair screening is usually performed by reagent suppliers. In multiplexed assays, the degree of NSB is proportional to the number of target analytes and antibodies.[12,178] Hence, more extensive characterization and careful selection of antibodies are needed. For example, Bosch *et al.* screened monoclonal antibodies in order to develop a multiplex LFA that could distinguish antigens from four serotypes of the Dengue and Zika viruses.[179] More detailed discussions are available in reviews on multiplexing POC diagnostics.[12,180]

Although protein/antibody-based LFAs have a rich history, LFAs based on different analyte/affinity molecules show promise for improving LFA performance. New affinity molecules, such as nanobodies, short peptides, and aptamers, can improve the LFA specificity and sensitivity due to their excellent reaction kinetics and selectivity for target analytes. A comparison of the available affinity molecules is provided in Table 4. A nanobody (*ca.* 15 kDa) is an antibody fragment (see Figure 2C(2)) that can be generated by recombinant methods. Due to their small size and concave shape, nanobodies can recognize and capture cryptic epitopes from antigens that are difficult to access with whole antibodies (usually 150 kDa).[12,181,182] Furthermore, due to the presence of fewer charged groups, nanobodies have fewer problems with cross reactions than whole antibodies.[183] Short peptides (5–20 amino acid residues) share a similar size and advantage as nanobodies (*ca.* 15 kDa). Aptamers are single-stranded oligonucleotides (usually 20–60 nucleotides, approximately 13–39 kDa) and are commonly selected by the systematic evolution of ligands by exponential enrichment process.[184] Compared with whole antibodies, nanobodies, and short peptides, aptamers are more thermally and chemically stable, have less batch-to-batch variability, better high-affinity kinetics, and lower dissociation constants, and they are easier to orient upon immobilization, thus providing more consistent and accurate assays.[185−187] Furthermore, antibodies, nanobodies, and short peptides can detect only immunogenic molecules (*i.e.*, proteins and haptens), whereas aptamers can detect any type of target regardless of whether it has immunogenic properties. Also, the better stability of aptamers compared to other affinity molecules enables aptamers to retain high affinity and specificity in different conditions.[185−187] Aptamers are, therefore, promising as substitutes for antibodies.[185−187] However, antibodies have the advantage of being able to detect larger targets than other affinity molecules due to their larger molecular size (*ca.* 150 kDa for antibodies *versus* usually <40 kDa for the other affinity molecules). In addition, the production method, cost, and commercialization can also affect the use of affinity molecules in applications. For example, aptamers are used less frequently than antibodies because aptamer selection and production are still being commercialized, whereas antibody generation has a much longer history

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

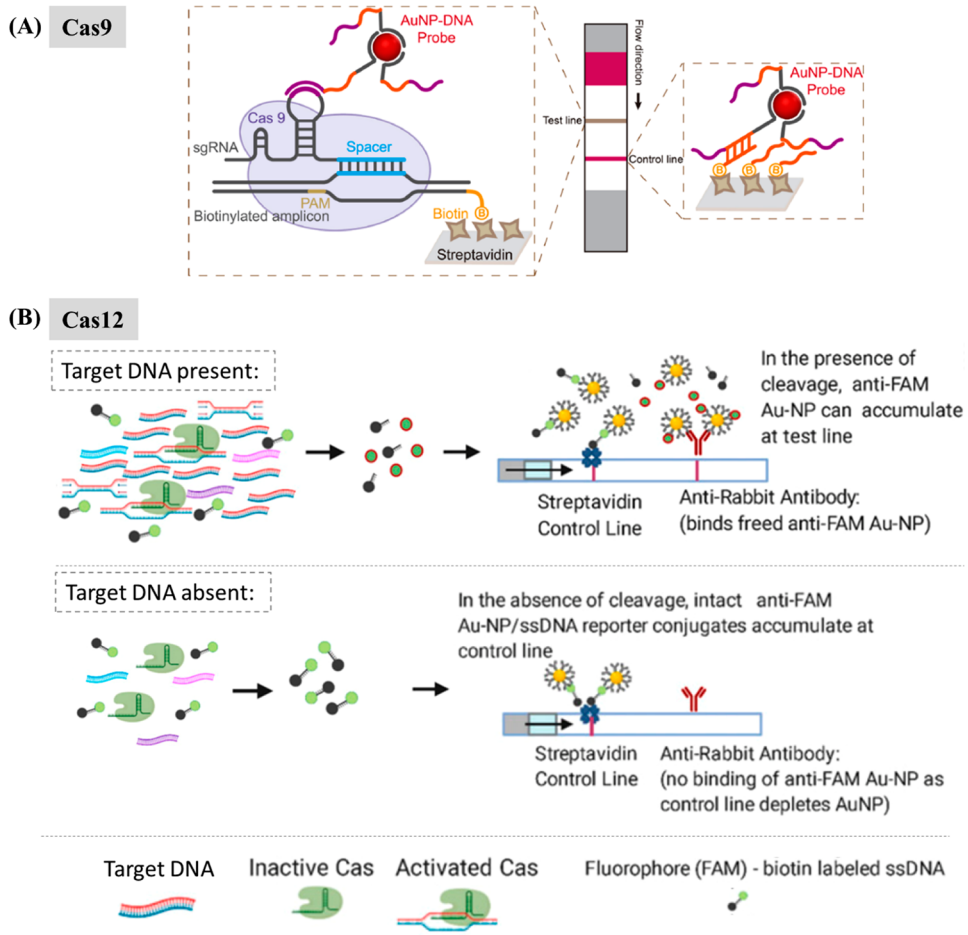


Figure 5. Lateral flow assay (LFA) readout formats in different Cas effector-mediated diagnostic platforms. (A) CRISPR/Cas9-mediated lateral flow nucleic acid assay[195] (sgRNA: single-guide RNA). (B) Cas12a-mediated DNA preamplification which cleaves biotin and FAM-labeled probes with an LFA readout.[198] In the absence of target DNAs, the LFA's control line will deplete the available gold nanoparticle (Au-NP) labels, leaving an empty test line.[198] (A) Reprinted from ref 195. Copyright 2020 American Chemical Society. (B) Adapted from ref 198, whose images are licensed under Creative Commons Attribution License 4.0 (CC BY).

(*ca.* 120 years for antibodies *versus ca.* 20 years for aptamers) and is already commercialized.[188] Comprehensive comparisons and reviews of the applications of affinity molecules have been provided in other work.[12,185−187]

Promising Highly Specific CRISPR/Cas Systems. CRISPR/Cas recognition is among the most promising, novel, and effective solutions to nonspecific nucleic acid diagnostic problems. CRISPR/Cas systems have a high binding specificity for target sequences and can even identify single nucleotide polymorphisms.[157] Multiple Cas effectors (Cas9, Cas12, Cas13) have been discovered since the first discovery of Cas9 in 2013[190,191] and have led to the 2020 Nobel Prize in Chemistry.[192] Table 5 briefly summarizes the characteristics of different Cas effectors and their reported diagnostic platforms. The working mechanisms and diagnostic applications of these CRISPR/Cas systems have been comprehensively reviewed in other work.[157,193] Briefly, Cas effectors use CRISPR RNA (crRNA) as a guide to recognize a target DNA/RNA and then perform target-dependent cleavage. The Cas12 and Cas13 effectors collaterally cleave the other DNA or RNA reporters in the system into small pieces, whereas Cas9 cleaves only the target dsDNA.

Substantial effort has been devoted to implementing CRISPR/Cas reactions in diagnostic platforms to achieve highly specific detection. Here, we limit our discussion to the LFA-based diagnostic platforms that hold promise for adaptation into future POC tests. For Cas9 effectors, the release of cleaved DNA is extremely slow and can take up to several hours.[194,195] To take advantage of this slow release, Wang *et al.* proposed a CRISPR/Cas9-mediated lateral flow nucleic acid assay (CASLFA) format (shown in Figure 5A) where the Cas9 effector was directly incorporated in the LFA.[195] In this CASLFA format, DNA-conjugated GNPs recognize a universal loop sequence from the crRNA (*i.e.*, CRISPR RNA) in a CRISPR/Cas9-DNA ternary complex, while the biotinylated DNA amplicon from that complex binds to the streptavidin-coated membrane to form a sandwich assay.[195] With amplified DNAs, the CASLFA format can reach a detection limit near the level of PCR (hundreds of copies of genomes per μL sample) with high specificity (100% for 110 clinical samples compared with the gold standard PCR results) within 1 h.[195] With regard to Cas12 and Cas13 effectors, the CRISPR/Cas reaction can simply be added after sample preamplification (by either PCR or isothermal methods) and before the LFA readout.[158,196,197] An example of a Cas12a-mediated diagnostic platform is shown in Figure 5B. In the Cas12a reaction, the presence of target sequences triggers the autocleavage of genome probes that are terminated by biotin and a fluorophore (FAM).[198] The LFAs are designed to detect the presence or absence of the autocleavage products. If not

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

cleaved, the control line with streptavidin depletes the biotin-linked sequence, leaving the test line blank.[198] If cleaved, the control line still binds with the biotin-linked sequence, while the cleaved FAM forms a sandwich assay on the test line.[198] As a result, the assay showed 100% sensitivity and specificity for 20 clinical samples that were previously characterized by PCR. This diagnostic principle has also been applied in other Cas12 and Cas13-mediated bioassays, such as DETECTR,[196] SHERLOCK,[158] and HUDSON.[197]

Note that these CRISPR/Cas-mediated diagnostic platforms can identify both DNA and RNA targets and are not limited by the target types listed in Table 5 when coupled with sample preamplification. This flexibility arises because the preamplification step can convert between nucleic acid types (transcription for DNA to RNA and reverse transcription (RT) for RNA to DNA). For example, by using RT-recombinase polymerase amplification (RT-RPA), the Cas13-mediated SHERLOCK test can detect both RNAs and DNAs.[199] Also, by designing different CRISPR/Cas systems paired with various end labels, multiplex diagnosis can be achieved.[158]

However, the assay time required for CRISPR/Cas recognition needs to be optimized, and the kinetics of target DNA/RNA recognition by Cas effectors and of nucleic acid cleavage require further study. For example, the reaction of Cas12b with target DNAs and the cleavage of probes takes approximately 30 min, which can add to the total assay time.[200] Thus, optimization to incorporate the CRISPR/Cas recognition step while maintaining a rapid test will be an important topic of further research. Also, as mentioned earlier, a "sample-to-answer" instrument that miniaturizes and automates all the reaction steps, including DNA/RNA extraction, amplification, CRISPR/Cas reaction, and readout, needs to be developed to achieve a future POC testing platform.

CONCLUSIONS AND PROSPECTS

Lateral flow assays continue to be among the most common POC tests globally due to their quick readout, low cost, and ease of use, with notable drawbacks being low sensitivity, low specificity, and lack of quantitation. Recent work on assay improvement and sample enrichment by preamplification is now addressing these remaining drawbacks and making LFAs competitive with more expensive and time-consuming laboratory tests (*e.g.*, ELISA and PCR). For instance, assay kinetics optimization and signal amplification (by chemical enhancement and reader use) enable LFAs to achieve ELISA-level sensitivities (pM–fM). Further improvements through approaches such as isothermal preamplification (direct or indirect) prior to the LFA readout are now poised to achieve even greater sensitivity. Furthermore, the specificity, which is of paramount importance to the LFA performance, can be enhanced through assay optimization and the identification and use of highly specific affinity molecules. With these improvements in sensitivity and specificity, clear proof of concept exists that ultrasensitive (aM) highly specific LFAs for POC diagnostics can be achieved. Nevertheless, the integration of sample treatment, preamplification, highly specific affinity molecule reactions (*e.g.*, CRISPR/Cas), and rapid LFA readout at an affordable cost are important challenges to be met. Ultimately, with the growing knowledge and tools reviewed here, we believe that these challenges will be met such that LFAs will soon achieve laboratory testing performance while maintaining their advantages for rapid and large-scale POC use.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.0c10035.

More tables and figures that summarize the nomenclature and descriptions, limits of detection of various signal-amplified LFAs, innovative work on assay kinetic optimization and chemical enhancement, and the comparison of sensitivity range increases imparted by various methods (PDF)

AUTHOR INFORMATION

Corresponding Author

John C. Bischof − *Department of Mechanical Engineering, University of Minnesota, Minneapolis, Minnesota 55455, United States; Department of Biomedical Engineering and Director, Institute of Engineering in Medicine, University of Minnesota, Minneapolis, Minnesota 55455, United States;* orcid.org/0000-0001-6726-7111; Email: bischof@umn.edu

Authors

Yilin Liu − *Department of Mechanical Engineering, University of Minnesota, Minneapolis, Minnesota 55455, United States;* orcid.org/0000-0002-8521-8943

Li Zhan − *Department of Mechanical Engineering, University of Minnesota, Minneapolis, Minnesota 55455, United States*

Zhenpeng Qin − *Department of Mechanical Engineering, Department of Bioengineering, and Center for Advanced Pain Studies, University of Texas at Dallas, Richardson, Texas 75080, United States; Department of Surgery, University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States;* orcid.org/0000-0003-3406-3045

James Sackrison − *3984 Hunters Hill Way, Minnetonka, Minnesota 55345, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsnano.0c10035

Notes

The authors declare the following competing financial interest(s): John Bischof is a founder and James Sackrison an employee of Vigilant Diagnostics. This company is focused on commercialization of thermal contrast amplification readers and lateral flow assays.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation (CBET- 2029474) and the Medtronic-Bakken Endowed Chair to J.C.B.

REFERENCES

(1) Peto, J.; Alwan, N. A.; Godfrey, K. M.; Burgess, R. A.; Hunter, D. J.; Riboli, E.; Romer, P.; Buchan, I.; Colbourn, T.; Costelloe, C.; Davey Smith, G.; et al. Universal Weekly Testing as the UK COVID-19 Lockdown Exit Strategy. *Lancet* **2020**, *395*, 1420−1421.

(2) Peto, J. Covid-19 Mass Testing Facilities Could End the Epidemic Rapidly. *BMJ.* **2020**, *368*, m1163.

(3) Peto, J.; Carpenter, J.; Smith, G. D.; Duffy, S.; Houlston, R.; Hunter, D. J.; McPherson, K.; Pearce, N.; Romer, P.; Sasieni, P.; et al. Weekly COVID-19 Testing with Household Quarantine and Contact Tracing Is Feasible and Would Probably End the Epidemic. *R. Soc. Open Sci.* **2020**, *7*, 200915.

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

(4) Larremore, D. B.; Wilder, B.; Lester, E.; Shehata, S.; Burke, J. M.; Hay, J. A.; Tambe, M.; Mina, M. J.; Parker, R. Test Sensitivity Is Secondary to Frequency and Turnaround Time for COVID-19 Surveillance. *Sci. Adv.* **2021**, *7*, eabd5393.
(5) Leon, D. A.; Shkolnikov, V. M.; Smeeth, L.; Magnus, P.; Pechholdová, M.; Jarvis, C. I. COVID-19: A Need for Real-Time Monitoring of Weekly Excess Deaths. *Lancet* **2020**, *395*, e81.
(6) Borst, A.; Box, A. T. A.; Fluit, A. C. False-Positive Results and Contamination in Nucleic Acid Amplification Assays: Suggestions for a Prevent and Destroy Strategy. *Eur. J. Clin. Microbiol. Infect. Dis.* **2004**, *23*, 289−299.
(7) Udugama, B.; Kadhiresan, P.; Kozlowski, H. N.; Malekjahani, A.; Osborne, M.; Li, V. Y. C.; Chen, H.; Mubareka, S.; Gubbay, J. B.; Chan, W. C. W. Diagnosing COVID-19: The Disease and Tools for Detection. *ACS Nano* **2020**, *14*, 3822−3835.
(8) Choi, J. R. Development of Point-of-Care Biosensors for COVID-19. *Front. Chem.* **2020**, *8*, 517.
(9) Pang, J.; Wang, M. X.; Ang, I. Y. H.; Tan, S. H. X.; Lewis, R. F.; Chen, J. I.-P.; Gutierrez, R. A.; Gwee, S. X. W.; Chua, P. E. Y.; Yang, Q.; et al. Potential Rapid Diagnostics, Vaccine and Therapeutics for 2019 Novel Coronavirus (2019-NCoV): A Systematic Review. *J. Clin. Med.* **2020**, *9*, 623.
(10) Grant, B. D.; Anderson, C. E.; Williford, J. R.; Alonzo, L. F.; Glukhova, V. A.; Boyle, D. S.; Weigl, B. H.; Nichols, K. P. A SARS-CoV-2 Coronavirus Nucleocapsid Antigen-Detecting Half-Strip Lateral Flow Assay Toward the Development of Point of Care Tests Using Commercially Available Reagents. *Anal. Chem.* **2020**, *92*, 11305−11309.
(11) Hu, J.; Wang, S. Q.; Wang, L.; Li, F.; Pingguan-Murphy, B.; Lu, T. J.; Xu, F. Advances in Paper-Based Point-of-Care Diagnostics. *Biosens. Bioelectron.* **2014**, *54*, 585−597.
(12) Soh, J. H.; Chan, H. M.; Ying, J. Y. Strategies for Developing Sensitive and Specific Nanoparticle-Based Lateral Flow Assays as Point-of-Care Diagnostic Device. *Nano Today* **2020**, *30*, 100831.
(13) Nguyen, V. T.; Song, S.; Park, S.; Joo, C. Recent Advances in High-Sensitivity Detection Methods for Paper-Based Lateral-Flow Assay. *Biosens. Bioelectron.* **2020**, *152*, 112015.
(14) Huang, X.; Aguilar, Z. P.; Xu, H.; Lai, W.; Xiong, Y. Membrane-Based Lateral Flow Immunochromatographic Strip with Nanoparticles as Reporters for Detection: A Review. *Biosens. Bioelectron.* **2016**, *75*, 166−180.
(15) Ye, H.; Liu, Y.; Zhan, L.; Liu, Y.; Qin, Z. Signal Amplification and Quantification on Lateral Flow Assays by Laser Excitation of Plasmonic Nanomaterials. *Theranostics* **2020**, *10*, 4359−4373.
(16) Jeong, H. W.; Heo, J. Y.; Park, J. S.; Kim, W. J. Effect of the Influenza Virus Rapid Antigen Test on a Physician's Decision to Prescribe Antibiotics and on Patient Length of Stay in the Emergency Department. *PLoS One* **2014**, *9*, e110978.
(17) Peci, A.; Winter, A. L.; King, E. C.; Blair, J.; Gubbay, J. B. Performance of Rapid Influenza Diagnostic Testing in Outbreak Settings. *J. Clin. Microbiol.* **2014**, *52*, 4309−4317.
(18) Lean, W. L.; Arnup, S.; Danchin, M.; Steer, A. C. Rapid Diagnostic Tests for Group a Streptococcal Pharyngitis: A Meta-Analysis. *Pediatrics* **2014**, *134*, 771−781.
(19) Wang, Y.; Louwagie, E.; Larkin, D.; Sankey, S.; Boulware, D. R.; Bischof, J. C. Improved Detection of Group A *Streptococcus* during Thermal Contrast Amplification *vs*. Visual Reading of Clinical Rapid Diagnostic Tests. *Anal. Methods* **2019**, *11*, 2013−2017.
(20) Adetunji, A. A.; Adewumi, M. O.; Michael, O. S.; Fayemiwo, S. A.; Ogunniyi, A.; Taiwo, B. O. Perspective Piece Rapid HIV Antigen-Antibody Assays and Detection of Acute HIV Infection in Sub-Saharan Africa. *Am. J. Trop. Med. Hyg.* **2019**, *101*, 285−286.
(21) Boulware, D. R.; Rolfes, M. A.; Rajasingham, R.; von Hohenberg, M.; Qin, Z.; Taseera, K.; Schutz, C.; Kwizera, R.; Butler, E. K.; Meintjes, G.; et al. Multisite Validation of Cryptococcal Antigen Lateral Flow Assay and Quantification by Laser Thermal Contrast. *Emerging Infect. Dis.* **2014**, *20*, 45−53.
(22) Azzam, I.; Pandori, M.; Sherych, L. Discontinue the Use of Antigen Testing in Skilled Nursing Facilities Until Further Notice; Nevada Department of Health and Human Services: Carson City, NV. http://dpbh.nv.gov/uploadedFiles/dpbhnvgov/content/Resources/Directive%20to%20Discontinue%20Use%20of%20Antigen%20POC_10.02.2020_ADA_Compliant.pdf (accessed 2020-10-09).
(23) Disease Burden of Influenza ; CDC: Atlanta, GA. https://www.cdc.gov/flu/about/burden/index.html?CDC_AA_refVal=https%3A%2F%2Fwww.cdc.gov%2Fflu%2Fabout%2Fdisease%2Fburden.htm (accessed 2020-12-08).
(24) Blaschke, A. J.; Shapiro, D. J.; Pavia, A. T.; Byington, C. L.; Ampofo, K.; Stockmann, C.; Hersh, A. L. A National Study of the Impact of Rapid Influenza Testing on Clinical Care in the Emergency Department. *J. Pediatric Infect. Dis. Soc.* **2014**, *3*, 112−118.
(25) Cruz, A. T.; Cazacu, A. C.; Greer, J. M.; Demmler, G. J. Rapid Assays for the Diagnosis of Influenza A and B Viruses in Patients Evaluated at a Large Tertiary Care Children's Hospital during Two Consecutive Winter Seasons. *J. Clin. Virol.* **2008**, *41*, 143−147.
(26) Bonner, A. B.; Monroe, K. W.; Talley, L. I.; Klasner, A. E.; Kimberlin, D. W. Impact of the Rapid Diagnosis of Influenza on Physician Decision-Making and Patient Management in the Pediatric Emergency Department: Results of a Randomized, Prospective, Controlled Trial. *Pediatrics* **2003**, *112*, 363−367.
(27) Balish, A.; Garten, R.; Klimov, A.; Villanueva, J. Analytical Detection of Influenza A(H3N2)v and Other A Variant Viruses from the USA by Rapid Influenza Diagnostic Tests. *Influenza Other Respir. Viruses* **2013**, *7*, 491−496.
(28) Van Tienen, C.; Rugebregt, S.; Scherbeijn, S.; Götz, H.; Geurts Van Kessel, C. The Performance of the Alere HIV Combo Point-of-Care Test on Stored Serum Samples; Useful for Detection of Early HIV-1 Infections? *Sex. Transm. Infect.* **2018**, *94*, 331−333.
(29) Laboratory Testing for the Diagnosis of HIV Infection: Updated Recommendations; CDC: Atlanta, GA. https://stacks.cdc.gov/view/cdc/23447 (accessed 2020-12-08).
(30) Böger, B.; Fachi, M. M.; Vilhena, R. O.; Cobre, A. F.; Tonin, F. S.; Pontarolo, R. Systematic Review with Meta-Analysis of the Accuracy of Diagnostic Tests for COVID-19. *Am. J. Infect. Control* **2021**, *49*, 21.
(31) Nagura-Ikeda, M.; Imai, K.; Tabata, S.; Miyoshi, K.; Murahara, N.; Mizuno, T.; Horiuchi, M.; Kato, K.; Imoto, Y.; Iwata, M.; et al. Clinical Evaluation of Self-Collected Saliva by Quantitative Reverse Transcription-PCR (RT-QPCR), Direct RT-QPCR, Reverse Transcription−Loop-Mediated Isothermal Amplification, and a Rapid Antigen Test To Diagnose COVID-19. *J. Clin. Microbiol.* **2020**, *58*, e01438.
(32) Bishop, J. D.; Hsieh, H. V.; Gasperino, D. J.; Weigl, B. H. Sensitivity Enhancement in Lateral Flow Assays: A Systems Perspective. *Lab Chip* **2019**, *19*, 2486−2499.
(33) Rodríguez, M. O.; Covián, L. B.; García, A. C.; Blanco-López, M. C. Silver and Gold Enhancement Methods for Lateral Flow Immunoassays. *Talanta* **2016**, *148*, 272−278.
(34) Cheng, N.; Song, Y.; Shi, Q.; Du, D.; Liu, D.; Luo, Y.; Xu, W.; Lin, Y. Au@Pd Nanopopcorn and Aptamer Nanoflower Assisted Lateral Flow Strip for Thermal Detection of Exosomes. *Anal. Chem.* **2019**, *91* (21), 13986−13993.
(35) Oliveira-Rodríguez, M.; López-Cobo, S.; Reyburn, H. T.; Costa-García, A.; López-Martín, S.; Yáñez-Mó, M.; Cernuda-Morollón, E.; Paschen, A.; Valés-Gómez, M.; Blanco-López, M. C. Development of a Rapid Lateral Flow Immunoassay Test for Detection of Exosomes Previously Enriched from Cell Culture Medium and Body Fluids. *J. Extracell. Vesicles* **2016**, *5*, 31803.
(36) López-Cobo, S.; Campos-Silva, C.; Moyano, A.; Oliveira-Rodríguez, M.; Paschen, A.; Yáñez-Mó, M.; Blanco-López, M. C.; Valés-Gómez, M. Immunoassays for Scarce Tumour-Antigens in Exosomes: Detection of the Human NKG2D-Ligand, MICA, in Tetraspanin-Containing Nanovesicles from Melanoma. *J. Nanobiotechnol.* **2018**, *16*, 1−12.

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

(37) Hwang, J.; Kwon, D.; Lee, S.; Jeon, S. Detection of Salmonella Bacteria in Milk Using Gold-Coated Magnetic Nanoparticle Clusters and Lateral Flow Filters. *RSC Adv.* **2016**, *6*, 48445−48448.
(38) Li, C. Z.; Vandenberg, K.; Prabhulkar, S.; Zhu, X.; Schneper, L.; Methee, K.; Rosser, C. J.; Almeide, E. Paper Based Point-of-Care Testing Disc for Multiplex Whole Cell Bacteria Analysis. *Biosens. Bioelectron.* **2011**, *26*, 4342−4348.
(39) Li, X.; Lu, D.; Sheng, Z.; Chen, K.; Guo, X.; Jin, M.; Han, H. A Fast and Sensitive Immunoassay of Avian Influenza Virus Based on Label-Free Quantum Dot Probe and Lateral Flow Test Strip. *Talanta* **2012**, *100*, 1−6.
(40) Thornton, C. R.; Groenhof, A. C.; Forrest, R.; Lamotte, R. A One-Step, Immunochromatographic Lateral Flow Device Specific to Rhizoctonia Solani and Certain Related Species, and Its Use to Detect and Quantify R. Solani in Soil. *Phytopathology* **2004**, *94*, 280−288.
(41) Poonlapdecha, W.; Seetang-Nun, Y.; Wonglumsom, W.; Tuitemwong, K.; Erickson, L. E.; Hansen, R. R.; Tuitemwong, P. Antibody-Conjugated Ferromagnetic Nanoparticles with Lateral Flow Test Strip Assay for Rapid Detection of Campylobacter Jejuni in Poultry Samples. *Int. J. Food Microbiol.* **2018**, *286*, 6−14.
(42) Gong, Y.; Zheng, Y.; Jin, B.; You, M.; Wang, J.; Li, X. J.; Lin, M.; Xu, F.; Li, F. A Portable and Universal Upconversion Nanoparticle-Based Lateral Flow Assay Platform for Point-of-Care Testing. *Talanta* **2019**, *201*, 126−133.
(43) Qin, Z.; Chan, W. C. W.; Boulware, D. R.; Akkin, T.; Butler, E. K.; Bischof, J. C. Significantly Improved Analytical Sensitivity of Lateral Flow Immunoassays by Using Thermal Contrast. *Angew. Chem., Int. Ed.* **2012**, *51*, 4358−4361.
(44) Wang, Y.; Qin, Z.; Boulware, D. R.; Pritt, B. S.; Sloan, L. M.; Gonzalez, I. J.; Bell, D.; Rees-Channer, R. R.; Chiodini, P.; Chan, W. C. W.; et al. Thermal Contrast Amplification Reader Yielding 8-Fold Analytical Improvement for Disease Detection with Lateral Flow Assays. *Anal. Chem.* **2016**, *88*, 11774−11782.
(45) Zhang, Y. J.; Chen, S.; Yu, Y. L.; Wang, J. H. A Miniaturized Photoacoustic Device with Laptop Readout for Point-of-Care Testing of Blood Glucose. *Talanta* **2020**, *209*, 120527.
(46) Zhao, Y.; Huang, Y.; Zhao, X.; McClelland, J. F.; Lu, M. Nanoparticle-Based Photoacoustic Analysis for Highly Sensitive Lateral Flow Assays. *Nanoscale* **2016**, *8*, 19204−19210.
(47) Song, S.; Choi, S.; Ryu, S.; Kim, S.; Kim, T.; Shin, J.; Jung, H. Il; Joo, C. Highly Sensitive Paper-Based Immunoassay Using Photothermal Laser Speckle Imaging. *Biosens. Bioelectron.* **2018**, *117*, 385−391.
(48) Ojaghi, A.; Pallapa, M.; Tabatabaei, N.; Rezai, P. High-Sensitivity Interpretation of Lateral Flow Immunoassays Using Thermophotonic Lock-in Imaging. *Sens. Actuators, A* **2018**, *273*, 189−196.
(49) Zhan, L.; Guo, S. Z.; Song, F.; Gong, Y.; Xu, F.; Boulware, D. R.; McAlpine, M. C.; Chan, W. C. W.; Bischof, J. C. The Role of Nanoparticle Design in Determining Analytical Performance of Lateral Flow Immunoassays. *Nano Lett.* **2017**, *17* (12), 7207−7212.
(50) Squires, T. M.; Messinger, R. J.; Manalis, S. R. Making It Stick: Convection, Reaction and Diffusion in Surface-Based Biosensors. *Nat. Biotechnol.* **2008**, *26*, 417−426.
(51) Zhan, L.; Granade, T.; Liu, Y.; Wei, X.; Youngpairoj, A.; Sullivan, V.; Johnson, J.; Bischof, J. Development and Optimization of Thermal Contrast Amplification Lateral Flow Immunoassays for Ultrasensitive HIV P24 Protein Detection. *Microsyst. Nanoeng.* **2020**, *6*, 54.
(52) Mosley, G. L.; Nguyen, P.; Wu, B. M.; Kamei, D. T. Development of Quantitative Radioactive Methodologies on Paper to Determine Important Lateral-Flow Immunoassay Parameters. *Lab Chip* **2016**, *16*, 2871−2881.
(53) Green, A. I.; Watsky, E. J.; Salzman, C. Drugs of Abuse. *Side Eff. Drugs Annu.* **1992**, *16*, 22−28.
(54) Mansfield, M. A. Nitrocellulose Membranes for Lateral Flow Immunoassays: A Technical Treatise. In *Lateral Flow Immunoassay*; Humana Press: New York, 2009; pp 1−19.
(55) Qian, S.; Bau, H. H. A Mathematical Model of Lateral Flow Bioreactions Applied to Sandwich Assays. *Anal. Biochem.* **2003**, *322*, 89−98.
(56) Moghadam, B. Y.; Connelly, K. T.; Posner, J. D. Two Orders of Magnitude Improvement in Detection Limit of Lateral Flow Assays Using Isotachophoresis. *Anal. Chem.* **2015**, *87*, 1009−1017.
(57) Karlsson, R.; Michaelsson, A.; Mattsson, L. Kinetic Analysis of Monoclonal Antibody-Antigen Interactions with a New Biosensor Based Analytical System. *J. Immunol. Methods* **1991**, *145*, 229−240.
(58) Malmborg, A.-C.; Michaëlsson, A.; Ohlin, M.; Jansson, B.; Borrebaeck, C. A. K. Real Time Analysis of Antibody-Antigen Reaction Kinetics. *Scand. J. Immunol.* **1992**, *35*, 643−650.
(59) Hardy, F.; Djavadi-Ohaniance, L.; Goldberg, M. E. Measurement of Antibody/Antigen Association Rate Constants in Solution by a Method Based on the Enzyme-Linked Immunosorbent Assay. *J. Immunol. Methods* **1997**, *200*, 155−159.
(60) Liang, T.; Robinson, R.; Houghtaling, J.; Fridley, G.; Ramsey, S. A.; Fu, E. Investigation of Reagent Delivery Formats in a Multivalent Malaria Sandwich Immunoassay and Implications for Assay Performance. *Anal. Chem.* **2016**, *88*, 2311−2320.
(61) Wetmur, J. G.; Fresco, J. DNA Probes: Applications of the Principles of Nucleic Acid Hybridization. *Crit. Rev. Biochem. Mol. Biol.* **1991**, *26*, 227−259.
(62) He, X.; Liu, Z.; Yang, Y.; Li, L.; Wang, L.; Li, A.; Qu, Z.; Xu, F. Sensitivity Enhancement of Nucleic Acid Lateral Flow Assays through a Physical-Chemical Coupling Method: Dissoluble Saline Barriers. *ACS Sensors* **2019**, *4*, 1691−1700.
(63) Sharma, A.; Tok, A. I. Y.; Lee, C.; Ganapathy, R.; Alagappan, P.; Liedberg, B. Magnetic Field Assisted Preconcentration of Biomolecules for Lateral Flow Assaying. *Sens. Actuators, B* **2019**, *285*, 431−437.
(64) Mashayekhi, F.; Le, A. M.; Nafisi, P. M.; Wu, B. M.; Kamei, D. T. Enhancing the Lateral-Flow Immunoassay for Detection of Proteins Using an Aqueous Two-Phase Micellar System. *Anal. Bioanal. Chem.* **2012**, *404*, 2057−2066.
(65) Lou, D.; Fan, L.; Ji, Y.; Gu, N.; Zhang, Y. A Signal Amplifying Fluorescent Nanoprobe and Lateral Flow Assay for Ultrasensitive Detection of Cardiac Biomarker Troponin I. *Anal. Methods* **2019**, *11*, 3506−3513.
(66) Welch, N. G.; Scoble, J. A.; Muir, B. W.; Pigram, P. J. Orientation and Characterization of Immobilized Antibodies for Improved Immunoassays (Review). *Biointerphases* **2017**, *12*, 02D301.
(67) Di Nardo, F.; Cavalera, S.; Baggiani, C.; Giovannoli, C.; Anfossi, L. Direct *vs* Mediated Coupling of Antibodies to Gold Nanoparticles: The Case of Salivary Cortisol Detection by Lateral Flow Immunoassay. *ACS Appl. Mater. Interfaces* **2019**, *11*, 32758−32768.
(68) Trilling, A. K.; Beekwilder, J.; Zuilhof, H. Antibody Orientation on Biosensor Surfaces: A Minireview. *Analyst* **2013**, *138*, 1619−1627.
(69) Saha, B.; Evers, T. H.; Prins, M. W. J. How Antibody Surface Coverage on Nanoparticles Determines the Activity and Kinetics of Antigen Capturing for Biosensing. *Anal. Chem.* **2014**, *86*, 8158−8166.
(70) Lee, J. H.; Seo, H. S.; Kwon, J. H.; Kim, H. T.; Kwon, K. C.; Sim, S. J.; Cha, Y. J.; Lee, J. Multiplex Diagnosis of Viral Infectious Diseases (AIDS, Hepatitis C, and Hepatitis A) Based on Point of Care Lateral Flow Assay Using Engineered Proteinticles. *Biosens. Bioelectron.* **2015**, *69*, 213−225.
(71) Tang, R. H.; Liu, L. N.; Zhang, S. F.; Li, A.; Li, Z. Modification of a Nitrocellulose Membrane with Cellulose Nanofibers for Enhanced Sensitivity of Lateral Flow Assays: Application to the Determination of Staphylococcus Aureus. *Microchim. Acta* **2019**, *186*, 831.
(72) Quesada-González, D.; Stefani, C.; González, I.; de la Escosura-Muñiz, A.; Domingo, N.; Mutjé, P.; Merkoçi, A. Signal Enhancement on Gold Nanoparticle-Based Lateral Flow Tests Using Cellulose Nanofibers. *Biosens. Bioelectron.* **2019**, *141*, 111407.
(73) Zhang, S. F.; Liu, L. N.; Tang, R. H.; Liu, Z.; He, X. C.; Qu, Z. G.; Li, F. Sensitivity Enhancement of Lateral Flow Assay by Embedding Cotton Threads in Paper. *Cellulose* **2019**, *26*, 8087−8099.

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

(74) Tsai, T. T.; Huang, T. H.; Chen, C. A.; Ho, N. Y. J.; Chou, Y. J.; Chen, C. F. Development a Stacking Pad Design for Enhancing the Sensitivity of Lateral Flow Immunoassay. *Sci. Rep.* **2018**, *8*, 17319.
(75) Lin, L. K.; Uzunoglu, A.; Stanciu, L. A. Aminolated and Thiolated PEG-Covered Gold Nanoparticles with High Stability and Antiaggregation for Lateral Flow Detection of Bisphenol A. *Small* **2018**, *14*, 1702828.
(76) Lu, X.; Mei, T.; Guo, Q.; Zhou, W.; Li, X.; Chen, J.; Zhou, X.; Sun, N.; Fang, Z. Improved Performance of Lateral Flow Immunoassays for Alpha-Fetoprotein and Vanillin by Using Silica Shell-Stabilized Gold Nanoparticles. *Microchim. Acta* **2019**, *186*, 2.
(77) Xu, S.; Zhang, G.; Fang, B.; Xiong, Q.; Duan, H.; Lai, W. Lateral Flow Immunoassay Based on Polydopamine-Coated Gold Nanoparticles for the Sensitive Detection of Zearalenone in Maize. *ACS Appl. Mater. Interfaces* **2019**, *11*, 31283−31290.
(78) Xu, H.; Chen, J.; Birrenkott, J.; Zhao, J. X.; Takalkar, S.; Baryeh, K.; Liu, G. Gold-Nanoparticle-Decorated Silica Nanorods for Sensitive Visual Detection of Proteins. *Anal. Chem.* **2014**, *86*, 7351−7359.
(79) Liu, X.; Yang, J.; Li, Q.; Wang, Y.; Wang, Y.; Li, G.; Shi, J.; Ding, P.; et al. A Strip Test for the Optical Determination of Influenza Virus H3 Subtype Using Gold Nanoparticle Coated Polystyrene Latex Microspheres. *Microchim. Acta* **2020**, *187*, 306.
(80) Serebrennikova, K.; Samsonova, J.; Osipov, A. Hierarchical Nanogold Labels to Improve the Sensitivity of Lateral Flow Immunoassay. *Nano-Micro Lett.* **2018**, *10*, 24.
(81) Qiu, W.; Baryeh, K.; Takalkar, S.; Chen, W.; Liu, G. Carbon Nanotube-Based Lateral Flow Immunoassay for Ultrasensitive Detection of Proteins: Application to the Determination of IgG. *Microchim. Acta* **2019**, *186*, 436.
(82) He, Y.; Zhang, S.; Zhang, X.; Baloda, M.; Gurung, A. S.; Xu, H.; Zhang, X.; Liu, G. Ultrasensitive Nucleic Acid Biosensor Based on Enzyme-Gold Nanoparticle Dual Label and Lateral Flow Strip Biosensor. *Biosens. Bioelectron.* **2011**, *26*, 2018−2024.
(83) Parolo, C.; de la Escosura-Muñiz, A.; Merkoçi, A. Enhanced Lateral Flow Immunoassay Using Gold Nanoparticles Loaded with Enzymes. *Biosens. Bioelectron.* **2013**, *40*, 412−416.
(84) Jiang, D.; Ni, D.; Rosenkrans, Z. T.; Huang, P.; Yan, X.; Cai, W. Nanozyme: New Horizons for Responsive Biomedical Applications. *Chem. Soc. Rev.* **2019**, *48*, 3683−3704.
(85) Loynachan, C. N.; Thomas, M. R.; Gray, E. R.; Richards, D. A.; Kim, J.; Miller, B. S.; Brookes, J. C.; Agarwal, S.; Chudasama, V.; McKendry, R. A.; et al. Platinum Nanocatalyst Amplification: Redefining the Gold Standard for Lateral Flow Immunoassays with Ultrabroad Dynamic Range. *ACS Nano* **2018**, *12*, 279−288.
(86) Zhang, J.; Yu, Q.; Qiu, W.; Li, K.; Qian, L.; Zhang, X.; Liu, G. Gold-Platinum Nanoflowers as a Label and as an Enzyme Mimic for Use in Highly Sensitive Lateral Flow Immunoassays: Application to Detection of Rabbit IgG. *Microchim. Acta* **2019**, *186*, 357.
(87) Gao, Z.; Ye, H.; Tang, D.; Tao, J.; Habibi, S.; Minerick, A.; Tang, D.; Xia, X. Platinum-Decorated Gold Nanoparticles with Dual Functionalities for Ultrasensitive Colorimetric *in Vitro* Diagnostics. *Nano Lett.* **2017**, *17*, 5572−5579.
(88) Anfossi, L.; Di Nardo, F.; Giovannoli, C.; Passini, C.; Baggiani, C. Increased Sensitivity of Lateral Flow Immunoassay for Ochratoxin A through Silver Enhancement. *Anal. Bioanal. Chem.* **2013**, *405*, 9859−9867.
(89) Shen, Y.; Shen, G. Signal-Enhanced Lateral Flow Immunoassay with Dual Gold Nanoparticle Conjugates for the Detection of Hepatitis B Surface Antigen. *ACS Omega* **2019**, *4*, 5083−5087.
(90) Rivas, L.; de la Escosura-Muñiz, A.; Serrano, L.; Altet, L.; Francino, O.; Sánchez, A.; Merkoçi, A. Triple Lines Gold Nanoparticle-Based Lateral Flow Assay for Enhanced and Simultaneous Detection of Leishmania DNA and Endogenous Control. *Nano Res.* **2015**, *8*, 3704−3714.
(91) Liu, C.; Jia, Q.; Yang, C.; Qiao, R.; Jing, L.; Wang, L.; Xu, C.; Gao, M. Lateral Flow Immunochromatographic Assay for Sensitive Pesticide Detection by Using Fe_3O_4 Nanoparticle Aggregates as Color Reagents. *Anal. Chem.* **2011**, *83*, 6778−6784.
(92) Ren, W.; Ballou, D. R.; FitzGerald, R.; Irudayaraj, J. Plasmonic Enhancement in Lateral Flow Sensors for Improved Sensing of *E. coli* O157:H7. *Biosens. Bioelectron.* **2019**, *126*, 324−331.
(93) Gao, Y.; Deng, X.; Wen, W.; Zhang, X.; Wang, S. Ultrasensitive Paper Based Nucleic Acid Detection Realized by Three-Dimensional DNA-AuNPs Network Amplification. *Biosens. Bioelectron.* **2017**, *92*, 529−535.
(94) Xu, Y.; Liu, Y.; Wu, Y.; Xia, X.; Liao, Y.; Li, Q. Fluorescent Probe-Based Lateral Flow Assay for Multiplex Nucleic Acid Detection. *Anal. Chem.* **2014**, *86*, 5611−5614.
(95) Lee, S. H.; Hwang, J.; Kim, K.; Jeon, J.; Lee, S.; Ko, J.; Lee, J.; Kang, M.; Chung, D. R.; Choo, J. Quantitative Serodiagnosis of Scrub Typhus Using Surface-Enhanced Raman Scattering-Based Lateral Flow Assay Platforms. *Anal. Chem.* **2019**, *91*, 12275−12282.
(96) Tran, V.; Walkenfort, B.; König, M.; Salehi, M.; Schlücker, S. Rapid, Quantitative, and Ultrasensitive Point-of-Care Testing: A Portable SERS Reader for Lateral Flow Assays in Clinical Chemistry. *Angew. Chem., Int. Ed.* **2019**, *58*, 442−446.
(97) Bhardwaj, J.; Sharma, A.; Jang, J. Vertical Flow-Based Paper Immunosensor for Rapid Electrochemical and Colorimetric Detection of Influenza Virus Using a Different Pore Size Sample Pad. *Biosens. Bioelectron.* **2019**, *126*, 36−43.
(98) Marquina, C.; De Teresa, J. M.; Serrate, D.; Marzo, J.; Cardoso, F. A.; Saurel, D.; Cardoso, S.; Freitas, P. P.; Ibarra, M. R. GMR Sensors and Magnetic Nanoparticles for Immuno-Chromatographic Assays. *J. Magn. Magn. Mater.* **2012**, *324*, 3495−3498.
(99) Lei, H.; Wang, K.; Ji, X.; Cui, D. Contactless Measurement of Magnetic Nanoparticles on Lateral Flow Strips Using Tunneling Magnetoresistance (TMR) Sensors in Differential Configuration. *Sensors* **2016**, *16*, 2130.
(100) Kim, J.; Mohamed, M. A. A.; Zagorovsky, K.; Chan, W. C. W. State of Diagnosing Infectious Pathogens Using Colloidal Nanomaterials. *Biomaterials* **2017**, *146*, 97−114.
(101) Sajid, M.; Kawde, A. N.; Daud, M. Designs, Formats and Applications of Lateral Flow Assay: A Literature Review. *J. Saudi Chem. Soc.* **2015**, *19*, 689−705.
(102) Wang, P.; Kricka, L. J. Current and Emerging Trends in Point-of-Care Technology and Strategies for Clinical Validation and Implementation. *Clin. Chem.* **2018**, *64*, 1439−1452.
(103) Jiang, N.; Ahmed, R.; Damayantharan, M.; Ünal, B.; Butt, H.; Yetisen, A. K. Lateral and Vertical Flow Assays for Point-of-Care Diagnostics. *Adv. Healthcare Mater.* **2019**, *8*, 1900244.
(104) Huang, Y.; Xu, T.; Wang, W.; Wen, Y.; Li, K.; Qian, L.; Zhang, X.; Liu, G. Lateral Flow Biosensors Based on the Use of Micro- and Nanomaterials: A Review on Recent Developments. *Microchim. Acta* **2020**, *187*, 70.
(105) Corstjens, P. L. A. M.; Van Lieshout, L.; Zuiderwijk, M.; Kornelis, D.; Tanke, H. J.; Deelder, A. M.; Van Dam, G. J. Up-Converting Phosphor Technology-Based Lateral Flow Assay for Detection of Schistosoma Circulating Anodic Antigen in Serum. *J. Clin. Microbiol.* **2008**, *46*, 171−176.
(106) Li, W.; Li, M.; Ge, S.; Yan, M.; Huang, J.; Yu, J. Battery-Triggered Ultrasensitive Electrochemiluminescence Detection on Microfluidic Paper-Based Immunodevice Based on Dual-Signal Amplification Strategy. *Anal. Chim. Acta* **2013**, *767*, 66−74.
(107) Shyu, R. H.; Shyu, H. F.; Liu, H. W.; Tang, S. S. Colloidal Gold-Based Immunochromatographic Assay for Detection of Ricin. *Toxicon* **2002**, *40*, 255−258.
(108) Parolo, C.; Sena Torralba, A.; Bergua, J. F.; Calucho, E.; Fuentes Chust, C.; Hu, L.; Rivas, L.; Álvarez Diduk, R.; Nguyen, E. P.; Cinti, S.; et al. Tutorial : Design and Fabrication of Nanoparticle-Based Lateral-Flow Immunoassays. *Nat. Protoc.* **2020**, *15*, 3788−3816.
(109) Hsieh, H. V.; Dantzler, J. L.; Weigl, B. H. Analytical Tools to Improve Optimization Procedures for Lateral Flow Assays. *Diagnostics* **2017**, *7*, 29.
(110) Wang, Y.; Fill, C.; Nugen, S. R. Development of Chemiluminescent Lateral Flow Assay for the Detection of Nucleic Acids. *Biosensors* **2012**, *2* (1), 32−42.

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

(111) Anderson, C. E.; Buser, J. R.; Fleming, A. M.; Strauch, E. M.; Ladd, P. D.; Englund, J.; Baker, D.; Yager, P. An Integrated Device for the Rapid and Sensitive Detection of the Influenza Hemagglutinin. *Lab Chip* **2019**, *19*, 885−896.
(112) Han, G. R.; Ki, H.; Kim, M. G. Automated, Universal, and Mass-Producible Paper-Based Lateral Flow Biosensing Platform for High-Performance Point-of-Care Testing. *ACS Appl. Mater. Interfaces* **2020**, *12*, 1885−1894.
(113) Yoon, C. H.; Cho, J. H.; Oh, H. Il; Kim, M. J.; Lee, C. W.; Choi, J. W.; Paek, S. H. Development of a Membrane Strip Immunosensor Utilizing Ruthenium as an Electro-Chemiluminescent Signal Generator. *Biosens. Bioelectron.* **2003**, *19*, 289−296.
(114) Du, D.; Wang, J.; Wang, L.; Lu, D.; Lin, Y. Integrated Lateral Flow Test Strip with Electrochemical Sensor for Quantification of Phosphorylated Cholinesterase: Biomarker of Exposure to Organophosphorus Agents. *Anal. Chem.* **2012**, *84*, 1380−1385.
(115) Sinawang, P. D.; Rai, V.; Ionescu, R. E.; Marks, R. S. Electrochemical Lateral Flow Immunosensor for Detection and Quantification of Dengue NS1 Protein. *Biosens. Bioelectron.* **2016**, *77*, 400−408.
(116) Wang, Y. Measurement and Application of Heat Generation from Gold Nanoparticle Systems under Laser Irradiation in Biomedicine. *Ph.D. Dissertation*; University of Minnesota, 2019.
(117) Mohd Hanafiah, K.; Arifin, N.; Bustami, Y.; Noordin, R.; Garcia, M.; Anderson, D. Development of Multiplexed Infectious Disease Lateral Flow Assays: Challenges and Opportunities. *Diagnostics* **2017**, *7*, 51.
(118) Kim, H.; Chung, D. R.; Kang, M. A New Point-of-Care Test for the Diagnosis of Infectious Diseases Based on Multiplex Lateral Flow Immunoassays. *Analyst* **2019**, *144*, 2460−2466.
(119) Anfossi, L.; Di Nardo, F.; Cavalera, S.; Giovannoli, C.; Baggiani, C. Multiplex Lateral Flow Immunoassay: An Overview of Strategies towards High-Throughput Point-of-Need Testing. *Biosensors* **2019**, *9*, 2.
(120) Chinnasamy, T.; Segerink, L. I.; Nystrand, M.; Gantelius, J.; Andersson Svahn, H. Point-of-Care Vertical Flow Allergen Microarray Assay: Proof of Concept. *Clin. Chem.* **2014**, *60*, 1209−1216.
(121) Shi, Z.; Tian, Y.; Wu, X.; Li, C.; Yu, L. A One-Piece Lateral Flow Impedimetric Test Strip for Label-Free Clenbuterol Detection. *Anal. Methods* **2015**, *7*, 4957−4964.
(122) Rissin, D. M.; Kan, C. W.; Campbell, T. G.; Howes, S. C.; Fournier, D. R.; Song, L.; Piech, T.; Patel, P. P.; Chang, L.; Rivnak, A. J.; et al. Single-Molecule Enzyme-Linked Immunosorbent Assay Detects Serum Proteins at Subfemtomolar Concentrations. *Nat. Biotechnol.* **2010**, *28*, 595−599.
(123) Kim, S. H.; Iwai, S.; Araki, S.; Sakakihara, S.; Iino, R.; Noji, H. Large-Scale Femtoliter Droplet Array for Digital Counting of Single Biomolecules. *Lab Chip* **2012**, *12*, 4986−4991.
(124) Wilson, D. H.; Rissin, D. M.; Kan, C. W.; Fournier, D. R.; Piech, T.; Campbell, T. G.; Meyer, R. E.; Fishburn, M. W.; Cabrera, C.; Patel, P. P.; et al. The Simoa HD-1 Analyzer: A Novel Fully Automated Digital Immunoassay Analyzer with Single-Molecule Sensitivity and Multiplexing. *J. Lab. Autom.* **2016**, *21*, 533−547.
(125) Yelleswarapu, V.; Buser, J. R.; Haber, M.; Baron, J.; Inapuri, E.; Issadore, D. Mobile Platform for Rapid Sub−Picogram-per-Milliliter, Multiplexed, Digital Droplet Detection of Proteins. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 4489−4495.
(126) Cohen, L.; Cui, N.; Cai, Y.; Garden, P. M.; Li, X.; Weitz, D. A.; Walt, D. R. Single Molecule Protein Detection with Attomolar Sensitivity Using Droplet Digital Enzyme-Linked Immunosorbent Assay. *ACS Nano* **2020**, *14* (8), 9491−9501.
(127) Akama, K.; Shirai, K.; Suzuki, S. Highly Sensitive Multiplex Protein Detection by Droplet-Free Digital ELISA. *Electron. Commun. Japan* **2019**, *102*, 43−47.
(128) Wu, C.; Garden, P. M.; Walt, D. R. Ultrasensitive Detection of Attomolar Protein Concentrations by Dropcast Single Molecule Assays. *J. Am. Chem. Soc.* **2020**, *142*, 12314−12323.
(129) Miller, B. S.; Bezinge, L.; Gliddon, H. D.; Huang, D.; Dold, G.; Gray, E. R.; Heaney, J.; Dobson, P. J.; Nastouli, E.; Morton, J. J. L.; et al. Spin-Enhanced Nanodiamond Biosensing for Ultrasensitive Diagnostics. *Nature* **2020**, *587*, 588−593.
(130) Yan, L.; Zhou, J.; Zheng, Y.; Gamson, A. S.; Roembke, B. T.; Nakayama, S.; Sintim, H. O. Isothermal Amplified Detection of DNA and RNA. *Mol. BioSyst.* **2014**, *10*, 970.
(131) Gill, P.; Ghaemi, A. Nucleic Acid Isothermal Amplification Technologies - A Review. *Nucleosides, Nucleotides Nucleic Acids* **2008**, *27*, 224−243.
(132) Zhao, Y.; Chen, F.; Li, Q.; Wang, L.; Fan, C. Isothermal Amplification of Nucleic Acids. *Chem. Rev.* **2015**, *115*, 12491−12545.
(133) Craw, P.; Balachandran, W. Isothermal Nucleic Acid Amplification Technologies for Point-of-Care Diagnostics: A Critical Review. *Lab Chip* **2012**, *12*, 2469−2486.
(134) Obande, G. A.; Singh, K. K. B. Current and Future Perspectives on Isothermal Nucleic Acid Amplification Technologies for Diagnosing Infections. *Infect. Drug Resist.* **2020**, *13*, 455−483.
(135) Nam, J.; Thaxton, C. S.; Mirkin, C. A. Nanoparticle-Based Bio-Bar Codes for the Ultrasensitive Detection of Proteins. *Science* **2003**, *301*, 1884−1887.
(136) Nam, J. M.; Stoeva, S. I.; Mirkin, C. A. Bio-Bar-Code-Based DNA Detection with PCR-Like Sensitivity. *J. Am. Chem. Soc.* **2004**, *126*, 5932−5933.
(137) Fang, Z.; Wu, W.; Lu, X.; Zeng, L. Lateral Flow Biosensor for DNA Extraction-Free Detection of Salmonella Based on Aptamer Mediated Strand Displacement Amplification. *Biosens. Bioelectron.* **2014**, *56*, 192−197.
(138) Nguyen, H. V.; Nguyen, V. D.; Nguyen, H. Q.; Chau, T. H. T.; Lee, E. Y.; Seo, T. S. Nucleic Acid Diagnostics on the Total Integrated Lab-on-a-Disc for Point-of-Care Testing. *Biosens. Bioelectron.* **2019**, *141*, 111466.
(139) Ye, X.; Xu, J.; Lu, L.; Li, X.; Fang, X.; Kong, J. Equipment-Free Nucleic Acid Extraction and Amplification on a Simple Paper Disc for Point-of-Care Diagnosis of Rotavirus A. *Anal. Chim. Acta* **2018**, *1018*, 78−85.
(140) Trieu, P. T.; Lee, N. Y. Paper-Based All-in-One Origami Microdevice for Nucleic Acid Amplification Testing for Rapid Colorimetric Identification of Live Cells for Point-of-Care Testing. *Anal. Chem.* **2019**, *91*, 11013−11022.
(141) Zhang, Y.; Zhang, L.; Sun, J.; Liu, Y.; Ma, X.; Cui, S.; Ma, L.; Xi, J. J.; Jiang, X. Point-of-Care Multiplexed Assays of Nucleic Acids Using Microcapillary-Based Loop-Mediated Isothermal Amplification. *Anal. Chem.* **2014**, *86*, 7057−7062.
(142) Yeh, E. C.; Fu, C. C.; Hu, L.; Thakur, R.; Feng, J.; Lee, L. P. Self-Powered Integrated Microfluidic Point-of-Care Low-Cost Enabling (SIMPLE) Chip. *Sci. Adv.* **2017**, *3*, e1501645.
(143) Lobato, I. M.; O'Sullivan, C. K. Recombinase Polymerase Amplification: Basics, Applications and Recent Advances. *TrAC, Trends Anal. Chem.* **2018**, *98*, 19−35.
(144) Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T. Loop-Mediated Isothermal Amplification of DNA. *Nucleic Acids Res.* **2000**, *28*, 63.
(145) Tomita, N.; Mori, Y.; Kanda, H.; Notomi, T. Loop-Mediated Isothermal Amplification (LAMP) of Gene Sequences and Simple Visual Detection of Products. *Nat. Protoc.* **2008**, *3*, 877−882.
(146) Jia, H.; Li, Z.; Liu, C.; Cheng, Y. Ultrasensitive Detection of MicroRNAs by Exponential Isothermal Amplification. *Angew. Chem., Int. Ed.* **2010**, *49*, 5498−5501.
(147) Xu, Y.; Niu, C.; Xiao, X.; Zhu, W.; Dai, Z.; Zou, X. Chemical-Oxidation Cleavage Triggered Isothermal Exponential Amplification Reaction for Attomole Gene-Specific Methylation Analysis. *Anal. Chem.* **2015**, *87*, 2945−2951.
(148) Abd El Wahed, A.; Patel, P.; Heidenreich, D.; Hufert, F. T.; Weidmann, M. Reverse Transcription Recombinase Polymerase Amplification Assay for the Detection of Middle East Respiratory Syndrome Coronavirus. *PLoS Curr.* **2013**, *5*, e8364.
(149) Amer, H. M.; Abd El Wahed, A.; Shalaby, M. A.; Almajhdi, F. N.; Hufert, F. T.; Weidmann, M. A New Approach for Diagnosis of Bovine Coronavirus Using a Reverse Transcription Recombinase

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

Polymerase Amplification Assay. *J. Virol. Methods* **2013**, *193*, 337−340.
(150) Liu, L.; Jiang, L.; Yu, Y.; Xia, X.; Pan, Y.; Yan, S.; Wang, Y. Rapid Diagnosis of *Vibrio owensii* Responsible for Shrimp Acute Hepatopancreatic Necrosis Disease with Isothermal Recombinase Polymerase Amplification Assay. *Mol. Cell. Probes* **2017**, *33*, 4−7.
(151) Daher, R. K.; Stewart, G.; Boissinot, M.; Boudreau, D. K.; Bergeron, M. G. Influence of Sequence Mismatches on the Specificity of Recombinase Polymerase Amplification Technology. *Mol. Cell. Probes* **2015**, *29*, 116−121.
(152) Liu, X.; Yan, Q.; Huang, J.; Chen, J.; Guo, Z.; Liu, Z.; Cai, L.; Li, R.; Wang, Y.; Yang, G.; et al. Influence of Design Probe and Sequence Mismatches on the Efficiency of Fluorescent RPA. *World J. Microbiol. Biotechnol.* **2019**, *35*, 95.
(153) Tan, E.; Erwin, B.; Dames, S.; Ferguson, T.; Buechel, M.; Irvine, B.; Voelkerding, K.; Niemz, A. Specific *versus* Nonspecific Isothermal DNA Amplification through Thermophilic Polymerase and Nicking Enzyme Activities. *Biochemistry* **2008**, *47*, 9987−9999.
(154) Wang, Q.; Zhou, Y.; Li, S.; Zhuo, C.; Xu, S.; Huang, L.; Yang, L.; Liao, K. Real-Time Fluorescence Loop Mediated Isothermal Amplification for the Detection of Acinetobacter Baumannii. *PLoS One* **2013**, *8*, e66406.
(155) Klein, M.; Eslami-Mossallam, B.; Arroyo, D. G.; Depken, M. Hybridization Kinetics Explains CRISPR-Cas Off-Targeting Rules. *Cell Rep.* **2018**, *22*, 1413−1423.
(156) Strohkendl, I.; Saifuddin, F. A.; Rybarski, J. R.; Finkelstein, I. J.; Russell, R. Kinetic Basis for DNA Target Specificity of CRISPR-Cas12a. *Mol. Cell* **2018**, *71*, 816−824.e3.
(157) Li, Y.; Li, S.; Wang, J.; Liu, G. CRISPR/Cas Systems towards Next-Generation Biosensing. *Trends Biotechnol.* **2019**, *37*, 730−743.
(158) Gootenberg, J. S.; Abudayyeh, O. O.; Kellner, M. J.; Joung, J.; Collins, J. J.; Zhang, F. Multiplexed and Portable Nucleic Acid Detection Platform with Cas13, Cas12a and Csm6. *Science* **2018**, *360*, 439−444.
(159) Mayboroda, O.; Katakis, I.; O'Sullivan, C. K. Multiplexed Isothermal Nucleic Acid Amplification. *Anal. Biochem.* **2018**, *545*, 20−30.
(160) Li, S.; Gu, Y.; Lyu, Y.; Jiang, Y.; Liu, P. Integrated Graphene Oxide Purification-Lateral Flow Test Strips (IGOP-LFTS) for Direct Detection of PCR Products with Enhanced Sensitivity and Specificity. *Anal. Chem.* **2017**, *89*, 12137−12144.
(161) Zhang, Y.; Heller, A. Reduction of the Nonspecific Binding of a Target Antibody and of Its Enzyme-Labeled Detection Probe Enabling Electrochemical Immunoassay of an Antibody through the 7 Pg/ML-100 Ng/ML (40 FM-400 PM) Range. *Anal. Chem.* **2005**, *77*, 7758−7762.
(162) Schramm, E. C.; Staten, N. R.; Zhang, Z.; Bruce, S. S.; Kellner, C.; Atkinson, J. P.; Kyttaris, V. C.; Tsokos, G. C.; Petri, M.; Sander Connolly, E.; et al. A Quantitative Lateral Flow Assay to Detect Complement Activation in Blood. *Anal. Biochem.* **2015**, *477*, 78−85.
(163) Brito-Santos, F.; Ferreira, M. d. F.; Trilles, L.; Muniz, M. d. M.; Veloso dos Santos, V. G.; Carvalho-Costa, F. A.; Meyer, W.; Wanke, B.; Lazéra, M. d. S. Preheating of Urine Improves the Specificity of Urinary Cryptococcal Antigen Testing Using the Lateral Flow Assay. *PLoS Neglected Trop. Dis.* **2017**, *11*, e0005304.
(164) Crannell, Z.; Castellanos-Gonzalez, A.; Nair, G.; Mejia, R.; White, A. C.; Richards-Kortum, R. Multiplexed Recombinase Polymerase Amplification Assay To Detect Intestinal Protozoa. *Anal. Chem.* **2016**, *88*, 1610−1616.
(165) Corstjens, P. L. A. M.; Zuiderwijk, M.; Nilsson, M.; Feindt, H.; Niedbala, R. S.; Tanke, H. J. Lateral-Flow and up-Converting Phosphor Reporters to Detect Single-Stranded Nucleic Acids in a Sandwich-Hybridization Assay. *Anal. Biochem.* **2003**, *312*, 191−200.
(166) Wilcox, T. M.; McKelvey, K. S.; Young, M. K.; Jane, S. F.; Lowe, W. H.; Whiteley, A. R.; Schwartz, M. K. Robust Detection of Rare Species Using Environmental DNA: The Importance of Primer Specificity. *PLoS One* **2013**, *8*, e59520.
(167) McCall, C. M.; Mosier, S.; Thiess, M.; Debeljak, M.; Pallavajjala, A.; Beierl, K.; Deak, K. L.; Datto, M. B.; Gocke, C. D.; Lin, M. T.; et al. False Positives in Multiplex PCR-Based Next-Generation Sequencing Have Unique Signatures. *J. Mol. Diagn.* **2014**, *16*, 541−549.
(168) Rohrman, B. A.; Leautaud, V.; Molyneux, E.; Richards-Kortum, R. R. A Lateral Flow Assay for Quantitative Detection of Amplified HIV-1 RNA. *PLoS One* **2012**, *7*, e45611.
(169) Edwards, K. A.; Baeumner, A. J. Optimization of DNA-Tagged Dye-Encapsulating Liposomes for Lateral-Flow Assays Based on Sandwich Hybridization. *Anal. Bioanal. Chem.* **2006**, *386*, 1335−1343.
(170) Choi, D. H.; Lee, S. K.; Oh, Y. K.; Bae, B. W.; Lee, S. D.; Kim, S.; Shin, Y. B.; Kim, M. G. A Dual Gold Nanoparticle Conjugate-Based Lateral Flow Assay (LFA) Method for the Analysis of Troponin I. *Biosens. Bioelectron.* **2010**, *25*, 1999−2002.
(171) Teerinen, T.; Lappalainen, T.; Erho, T. A Paper-Based Lateral Flow Assay for Morphine. *Anal. Bioanal. Chem.* **2014**, *406*, 5955−5965.
(172) De Puig, H.; Bosch, I.; Carré-Camps, M.; Hamad-Schifferli, K. Effect of the Protein Corona on Antibody-Antigen Binding in Nanoparticle Sandwich Immunoassays. *Bioconjugate Chem.* **2017**, *28*, 230−238.
(173) Zhang, P.; Liu, X.; Wang, C.; Zhao, Y.; Hua, F.; Li, C.; Yang, R.; Zhou, L. Evaluation of Up-Converting Phosphor Technology-Based Lateral Flow Strips for Rapid Detection of Bacillus Anthracis Spore, Brucella Spp., and Yersinia Pestis. *PLoS One* **2014**, *9*, e105305.
(174) Kairdolf, B. A.; Mancini, M. C.; Smith, A. M.; Nie, S. Minimizing Nonspecific Cellular Binding of Quantum Dots with Hydroxyl-Derivatized Surface Coatings. *Anal. Chem.* **2008**, *80*, 3029−3034.
(175) Brooks, B. D.; Albertson, A. E.; Jones, J. A.; Speare, J. O.; Lewis, R. V. Efficient Screening of High-Signal and Low-Background Antibody Pairs in the Bio-Bar Code Assay Using Prion Protein as the Target. *Anal. Biochem.* **2008**, *382*, 60−62.
(176) Bembenek, M. E.; Burkhardt, A.; Ma, J.; Li, Z.; Loke, H.-k.; Wu, D.; Xu, Q.; Tayber, O.; Xie, L.; Li, P.; et al. Determination of Complementary Antibody Pairs Using Protein A Capture with the AlphaScreen Assay Format. *Anal. Biochem.* **2011**, *408*, 321−327.
(177) Wu, D.; Dumont Milutinovic, M.; Walt, D. R. Single Molecule Array (Simoa) Assay with Optimal Antibody Pairs for Cytokine Detection in Human Serum Samples †. *Analyst* **2015**, *140*, 6277−6282.
(178) Yagn, Y.; Kheiralla, Y.; Anderson, B.; Cobb, K. US Patent-Diagnostic Assays Including Multiplexed Lateral Flow Immunoassays with Quantum Dots, March 30, 2008.
(179) Bosch, I.; De Puig, H.; Hiley, M.; Carré-Camps, M.; Perdomo-Celis, F.; Narváez, C. F.; Salgado, D. M.; Senthoor, D.; O'Grady, M.; Phillips, E.; et al. Rapid Antigen Tests for Dengue Virus Serotypes and Zika Virus in Patient Serum. *Sci. Transl. Med.* **2017**, *9*, eaan1589.
(180) Mahmoudi, T.; de la Guardia, M.; Baradaran, B. Lateral Flow Assays towards Point-of-Care Cancer Detection: A Review of Current Progress and Future Trends. *TrAC, Trends Anal. Chem.* **2020**, *125*, 115842.
(181) Lauwereys, M.; Ghahroudi, M. A.; Desmyter, A.; Kinne, J.; Hölzer, W.; De Genst, E.; Wyns, L.; Muyldermans, S. Potent Enzyme Inhibitors Derived from Dromedary Heavy-Chain Antibodies. *EMBO J.* **1998**, *17*, 3512−3520.
(182) De Genst, E.; Silence, K.; Decanniere, K.; Conrath, K.; Loris, R.; Kinne, J.; Muyldermans, S.; Wyns, L. Molecular Basis for the Preferential Cleft Recognition by Dromedary Heavy-Chain Antibodies. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 4586−4591.
(183) Javadzadeh, Y.; Hamedeyaz, S. Floating Drug Delivery Systems for Eradication of Helicobacter Pylori in Treatment of Peptic Ulcer Disease. In *Trends in Helicobacter pylori Infection*; InTech: London, 2014.
(184) Tuerk, C.; Gold, L. Systematic Evolution of Ligands by Exponential Enrichment: RNA Ligands to Bacteriophage T4 DNA Polymerase. *Science* **1990**, *249* (4968), 505−510.
(185) Tombelli, S.; Minunni, M.; Mascini, M. Analytical Applications of Aptamers. *Biosens. Bioelectron.* **2005**, *20*, 2424−2434.

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX

(186) Chen, A.; Yang, S. Replacing Antibodies with Aptamers in Lateral Flow Immunoassay. *Biosens. Bioelectron.* **2015**, *71*, 230−242.
(187) Jayasena, S. D. Aptamers: An Emerging Class of Molecules That Rival Antibodies in Diagnostics. *Clin. Chem.* **1999**, *45*, 1628−1650.
(188) Toh, S. Y.; Citartan, M.; Gopinath, S. C. B.; Tang, T. H. Aptamers as a Replacement for Antibodies in Enzyme-Linked Immunosorbent Assay. *Biosens. Bioelectron.* **2015**, *64*, 392−403.
(189) Hermanson, G. T. (Strept)Avidin−Biotin Systems. In *Bioconjugate Techniques*; Elsevier: Amsterdam, 2013; pp 465−505.
(190) Mali, P.; Yang, L.; Esvelt, K. M.; Aach, J.; Guell, M.; DiCarlo, J. E.; Norville, J. E.; Church, G. M. RNA-Guided Human Genome Engineering *via* Cas9. *Science* **2013**, *339*, 823−826.
(191) Cong, L.; Ran, F. A.; Cox, D.; Lin, S.; Barretto, R.; Habib, N.; Hsu, P. D.; Wu, X.; Jiang, W.; Marraffini, L. A.; et al. Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* **2013**, *339*, 819−823.
(192) Uyhazi, K. E.; Bennett, J. A CRISPR View of the 2020 Nobel Prize in Chemistry. *J. Clin. Invest.* **2021**, *131*, 145214.
(193) Mustafa, M. I.; Makhawi, A. M. SHERLOCK and DETECTR: CRISPR-Cas Systems as Potential Rapid Diagnostic Tools for Emerging Infectious Diseases. *J. Clin. Microbiol.* **2020**, DOI: 10.1128/JCM.00745-20.
(194) Ma, H.; Tu, L. C.; Naseri, A.; Huisman, M.; Zhang, S.; Grunwald, D.; Pederson, T. CRI SPR-Cas9 Nuclear Dynamics and Target Recognition in Living Cells. *J. Cell Biol.* **2016**, *214*, 529−537.
(195) Wang, X.; Xiong, E.; Tian, T.; Cheng, M.; Lin, W.; Wang, H.; Zhang, G.; Sun, J.; Zhou, X. Clustered Regularly Interspaced Short Palindromic Repeats/Cas9-Mediated Lateral Flow Nucleic Acid Assay. *ACS Nano* **2020**, *14*, 2497−2508.
(196) Broughton, J. P.; Deng, X.; Yu, G.; Fasching, C. L.; Servellita, V.; Singh, J.; Miao, X.; Streithorst, J. A.; Granados, A.; Sotomayor-Gonzalez, A.; et al. CRISPR−Cas12-Based Detection of SARS-CoV-2. *Nat. Biotechnol.* **2020**, *38*, 870−874.
(197) Myhrvold, C.; Freije, C. A.; Gootenberg, J. S.; Abudayyeh, O. O.; Metsky, H. C.; Durbin, A. F.; Kellner, M. J.; Tan, A. L.; Paul, L. M.; Parham, L. A.; et al. Field-Deployable Viral Diagnostics Using CRISPR-Cas13. *Science* **2018**, *360*, 444−448.
(198) Lee, R. A.; De Puig, H.; Nguyen, P. Q.; Angenent-Mari, N. M.; Donghia, N. M.; McGee, J. P.; Dvorin, J. D.; Klapperich, C. M.; Pollock, N. R.; Collins, J. J. Ultrasensitive CRISPR-Based Diagnostic for Field-Applicable Detection of Plasmodium Species in Symptomatic and Asymptomatic Malaria. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 25722−25731.
(199) Gootenberg, J. S.; Abudayyeh, O. O.; Lee, J. W.; Essletzbichler, P.; Dy, A. J.; Joung, J.; Verdine, V.; Donghia, N.; Daringer, N. M.; Freije, C. A.; Myhrvold, C.; et al. Nucleic Acid Detection with CRISPR-Cas13a/C2c2. *Science* **2017**, *356*, 438−442.
(200) Li, L.; Li, S.; Wu, N.; Wu, J.; Wang, G.; Zhao, G.; Wang, J. HOLMESv2: A CRISPR-Cas12b-Assisted Platform for Nucleic Acid Detection and DNA Methylation Quantitation. *ACS Synth. Biol.* **2019**, *8*, 2228−2237.
(201) Qiu, X. Y.; Zhu, L. Y.; Zhu, C. S.; Ma, J. X.; Hou, T.; Wu, X. M.; Xie, S. S.; Min, L.; Tan, D. A.; Zhang, D. Y.; et al. Highly Effective and Low-Cost MicroRNA Detection with CRISPR-Cas9. *ACS Synth. Biol.* **2018**, *7*, 807−813.
(202) Pardee, K.; Green, A. A.; Takahashi, M. K.; Braff, D.; Lambert, G.; Lee, J. W.; Ferrante, T.; Ma, D.; Donghia, N.; Fan, M.; et al. Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components. *Cell* **2016**, *165*, 1255−1266.
(203) Zhang, Y.; Qian, L.; Wei, W.; Wang, Y.; Wang, B.; Lin, P.; Liu, W.; Xu, L.; Li, X.; Liu, D.; et al. Paired Design of DCas9 as a Systematic Platform for the Detection of Featured Nucleic Acid Sequences in Pathogenic Strains. *ACS Synth. Biol.* **2017**, *6*, 211−216.
(204) Huang, M.; Zhou, X.; Wang, H.; Xing, D. Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 Triggered Isothermal Amplification for Site-Specific Nucleic Acid Detection. *Anal. Chem.* **2018**, *90*, 2193−2200.
(205) Chen, J. S.; Ma, E.; Harrington, L. B.; Da Costa, M.; Tian, X.; Palefsky, J. M.; Doudna, J. A. CRISPR-Cas12a Target Binding Unleashes Indiscriminate Single-Stranded DNase Activity. *Science* **2018**, *360*, 436−439.
(206) Li, S.-Y.; Cheng, Q.-X.; Wang, J.-M.; Li, X.-Y.; Zhang, Z.-L.; Gao, S.; Cao, R.-B.; Zhao, G.-P.; Wang, J. CRISPR-Cas12a-Assisted Nucleic Acid Detection. *Cell Discovery* **2018**, *4*, 20.
(207) Kellner, M. J.; Koob, J. G.; Gootenberg, J. S.; Abudayyeh, O. O.; Zhang, F. SHERLOCK: Nucleic Acid Detection with CRISPR Nucleases. *Nat. Protoc.* **2019**, *14*, 2986−3012.

https://dx.doi.org/10.1021/acsnano.0c10035
ACS Nano XXXX, XXX, XXX−XXX