CHAPTER FOURTEEN

# Sensitive Protein Detection and Quantification in Paper-Based Microfluidics for the Point of Care

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## Contents



## Abstract

The design of appropriate diagnostic assays for the point of care requires development of suitable biosensors, detection methods, and diagnostic platforms for sensitive, quantitative detection of biological analytes. Protein targets in particular are especially challenging to detect quantitatively and sensitively due to the lack of amplification strategies akin to nucleic acid amplification. However, recent advances in transducer and biosensor design, new detection labels, and paper-based microfluidics may realize the goal of sensitive, fast, portable, and low-cost protein detection.

In this review, we discuss the biochemistry, optics, and engineering advances that may be leveraged to design such a sensitive protein diagnostic assay. The binding

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*Methods in Enzymology*, Volume 589
ISSN 0076-6879
http://dx.doi.org/10.1016/bs.mie.2017.01.018

kinetics, mechanisms of binding in porous networks, and potential transducers are explained in detail. We discuss the relative merits of various optical detection strategies, potential detection labels, optical readout approaches, and image-processing techniques that are amenable to point-of-care use. To conclude, we present a systematic analysis of potential approaches to enhance the sensitivity of paper-based assays. The assay development framework presented here provides bioassay developers a strategy to methodically enhance the sensitivity and point-of-care suitability of protein diagnostics.

## 1. INTRODUCTION

Diagnosing disease at the point of care remains challenging, especially in resource-limited settings, due to a dearth of appropriate, sensitive, low-cost, and easy-to-use diagnostic assays. Lateral flow assays are a suitable diagnostic platform for the point of care due to their low cost, ease of use, and portability, but one reason that their widespread use is hindered is their poor analytical sensitivity. This drawback is especially acute for protein diagnostics, as there exists no direct, general protein amplification analogs akin to polymerase chain reaction for nucleic acids. However, recent interdisciplinary advances in protein design, biomolecule production, and semiconductor manufacturing may be leveraged to bridge the gap in sensitivity, thereby enabling the design of rapid, sensitive, low-cost, and point-of-care protein diagnostic assays.

In this review, we explore key concepts associated with point-of-care protein detection and quantification. This review is structured to provide assay development guidelines and is neither meant to be a history of protein detection nor an exhaustive list of references, which are available in several excellent reviews published recently (Fu, 2014; Griffin, Williams, Stratis-Cullum, & Vo-Dinh, 2003; Janssen, Knez, Spasic, & Lammertyn, 2013; Yetisen et al., 2013). Rather, we discuss herein strategies for developing rapid, ultrasensitive protein assays while acknowledging the often-conflicting assay optimization goals of sensitivity, cost, portability, and speed. Toward this end, we explore the suitability of paper-based assays to realize these goals, summarizing the kinetics and biochemistry concepts underlying the selection of protein epitope-binding elements, optical labels, and detectors. Finally, we explicate strategies to enhance the sensitivity of bioassays. Together, the insights contained herein ought to provide a framework for bioassay developers to methodically enhance the sensitivity and point-of-care suitability of protein diagnostics.

## 2. PAPER-BASED DEVICES AND SANDWICH LATERAL FLOW ASSAYS

Paper-based assays use thin sheets of porous media, such as nitrocellulose, cellulose, or glass fiber, to manipulate and process small volumes of fluids. Such assays are especially conducive to point-of-care testing of biological analytes due to their low cost, portability, and ease of use. Lateral flow assays are the simplest paper-based devices and consist of strips of nitrocellulose that wick fluid based on capillary flow without requiring pumps or additional machinery. A sample pad is usually present at the "input" end, which is often a glass fiber pad onto which a sample is added. The sample then elutes into a conjugate pad, which is typically also a glass fiber material, in which reagents for the assay are stored in dry form and are rehydrated by application of a sample to the sample pad (Fig. 1A). A wicking or collection pad, often made of cellulose, is typically present at the end of the device opposite from the "input" end to draw fluids through the rest of the device. A typical lateral flow assay has a test line and a control line orthogonal to the flow direction, both of which are zones in which capture agents have been deposited on the membrane. Presence of the target analyte and adequate fluid delivery, along with rehydration of assay chemistry, is indicated by a change in color or luminescence of the test and control lines, respectively. Pregnancy tests, which detect human chorionic gonadotropin, are examples of lateral flow assays commonly used for protein detection at the point of care.

Despite the commercial success of the urine-based pregnancy test, many other assays require more complex manipulation of the biological sample to enable clinically useful sensitivity in the detection of an analyte. Platforms such as the two-dimensional paper network (2DPN) format or microfluidic paper-based analytical device (μPAD) format enable the design of these more complex assays through selective or sequential delivery of reagents or fluids (Fig. 1) (Fu, Kauffman, Lutz, & Yager, 2010; Martinez, Phillips, Butte, & Whitesides, 2007; Martinez, Phillips, Whitesides, & Carrilho, 2010). Key concepts associated with manufacturing 2DPN and μPAD devices are explicated in several recent reviews and papers (López-Marzo et al., 2016; Martinez et al., 2010; O'Farrell et al., 2009; Parolo, Medina-Sánchez, de la Escosura-Muñiz, & Merkoçi, 2013; Posthuma-Trumpie, Korf, & Van Amerongen, 2009). Briefly, the paper itself may be cut via laser cutting, mechanical shearing, or slicing; reagents may be deposited via

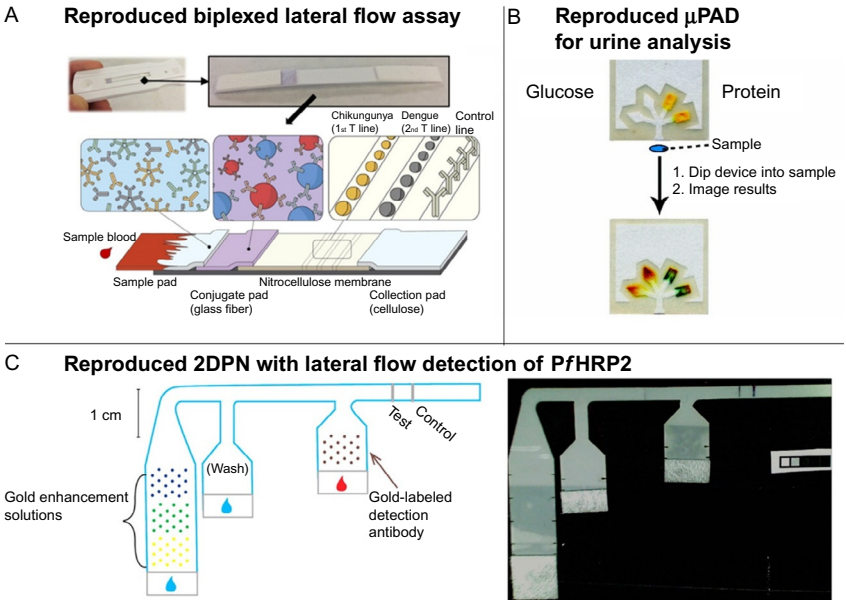


**Fig. 1** Examples of paper-based assays. (A) Schematic and photograph of a multiplexed lateral flow assay for differentiating febrile illnesses. (B) Photograph and usage of a microfluidic paper analytical device for the detection of glucose and protein in urine. (C) Schematics and photograph of a two-dimensional paper network coupled with lateral flow readout of *P. falciparum* HRP2. *Images reprinted with permission Fridley, G. E., Le, H., & Yager, P. (2014). Highly sensitive immunoassay based on controlled rehydration of patterned reagents in a 2-dimensional paper network.* Analytical Chemistry, 86*(13), 6447–6453. http://doi.org/10.1021/ac500872j; Lee, S., Mehta, S., & Erickson, D. (2016). Two-color lateral flow assay for multiplex detection of causative agents behind acute febrile illnesses.* Analytical Chemistry. *http://doi.org/10.1021/acs.analchem.6b01828; Martinez, A. W., Phillips, S. T., Carrilho, E., Thomas, S. W., Sindi, H., & Whitesides, G. M. (2008). Simple telemedicine for developing regions: Camera phones and paper-based microfluidic devices for real-time, off-site diagnosis.* Analytical Chemistry, 80*(10), 3699–3707. http://doi.org/10.1021/ac800112r. Copyright 2016, 2008, and 2014 American Chemical Society.*

patterning, printing, or striping; and flow may be controlled through valves, wax deposition, or material layering. These strategies can also be combined with 3D printing, casting, or molding when fabricating a device housing. Such 2DPN and μPAD systems are often then coupled with lateral flow assays for quantitative readout of the analyte concentration.

Traditional lateral flow assays are initiated by the application of a sample onto the device, where the sample passes through a conjugate pad and rehydrates the detection complex. The complex then flows downstream

and binds to the capture element deposited at the test line. This type of assay is a sandwich assay, as the macromolecule analyte is sandwiched between two binding elements at different epitopes. The detection element in this assay stack can be either directly or indirectly coupled to an optical label for visual readout. Adequate flow and performance of assay reagents, including the detection element and optical labeling chemistry, is confirmed by the development of signal at the control line. As the major components involved in binding of the analyte, the ligands responsible for the capture and detection of binding events play a significant role in overall assay sensitivity. The success of these ligands is strongly influenced by their binding thermodynamics and kinetics, including physical and chemical properties ranging from steric effects to isoelectric points (Holstein, 2015).

Key benefits derived from the porous nature of the membranes used in paper-based diagnostics, as compared to traditional microfluidic devices, include potentially improved analytical performance, improved ease of use by fewer user steps, reduced cost, and electricity-free usage. This contrasts with traditional microfluidic devices that often require electronic pumps and costly fabrication. Paper-based diagnostics eliminate the need for complex machinery, pumps, and other electrical equipment by manipulating fluids with capillary forces through the pores of the paper membrane (Martinez et al., 2010). The porous substrate also increases the surface area-to-volume ratio of the capture and control regions, thereby enabling localized reagent deposition (Yetisen et al., 2013). The assay's limit of detection and analytical sensitivity are thus improved by the increase in the relative concentration of the capture, detection, and optical elements. Since the surface area-to-volume ratio (typically 50–200 times greater than that of the bulk material) is inversely related to the pore size of the paper substrate, assay performance may be optimized by simply tuning the physical properties of the paper substrate used (EMD Millipore, 2013; Martinez et al., 2010; Yetisen et al., 2013). These advantages may be realized in 2DPNs or microfluidic paper-based devices to implement controlled fluid manipulation, reagent delivery, and rinse steps to further enhance assay performance and reduce user steps (Fu et al., 2010; Grant, Smith, Karvonen, & Richards-Kortum, 2016; Martinez et al., 2010). Paper-based diagnostics are thus an inherently suitable format for where they are needed the most (Fu et al., 2010; Grant et al., 2016; Martinez et al., 2010; Ngom, Guo, Wang, & Bi, 2010; Yetisen et al., 2013), such as for point-of-care protein detection.

## 3. ASSAY ANALYSIS FRAMEWORK

Assay developers typically have to balance the need to optimize an assay's sensitivity, cost, portability, and speed to suit a particular clinical usage scenario. These needs are discussed in varying levels of detail in publications that document the assay development process (Borysiak et al., 2016; Fu, 2014; Holstein, 2015; Holstein, Griffin, Hong, & Sampson, 2015; Rohrman & Richards-Kortum, 2012). Briefly, some factors to consider include assessing the envisioned usage scenario, such as identifying the end user, environment, and clinical workflow; identifying clinically relevant levels of analyte sensitivity and specificity; determining the appropriate form factor and user experience; determining what analyte(s), sample, and sample volume are needed to attain the intended clinical sensitivity and specificity; and determining the adequate analytical sensitivity and specificity that yields the desired clinical sensitivity and specificity. This distinction between analytical and clinical sensitivity is critical as the two differ considerably in scope: while the analytical sensitivity refers to the slope of the standard curve, clinical sensitivity refers to the ratio of true positives to the total number of diseased individuals in a sample population (Borysiak et al., 2016). The clinical sensitivity of an assay is affected by factors as diverse as the amount of analyte present and assay inhibitors present in real patient samples. Throughout the assay design process, it is important to engage with the intended customers, end users, clinicians, and patients to ensure that the assay meets a practical need. Moreover, it is equally essential to establish a priori design criteria, design constraints, and performance metrics so that the assay may be sufficiently validated.

In order to compare the performance of different assays and assess whether an assay meets performance metrics, appropriate quantitative and statistical methods are necessary to analyze lateral flow assays with statistical validity and reliability (Borysiak et al., 2016; Holstein, Griffin, et al., 2015). Briefly, assay developers assess the extent to which an assay is quantitative by creating a standard curve (binding isotherm) that plots an appropriately defined signal-to-noise ratio against the target analyte concentration, which will typically be fitted to a four-parameter logistic function via nonlinear regression (Fig. 2). The limit of detection (lower limit of detection) should then be defined as the lowest quantified concentration based on the distribution of signal-to-noise ratios of the

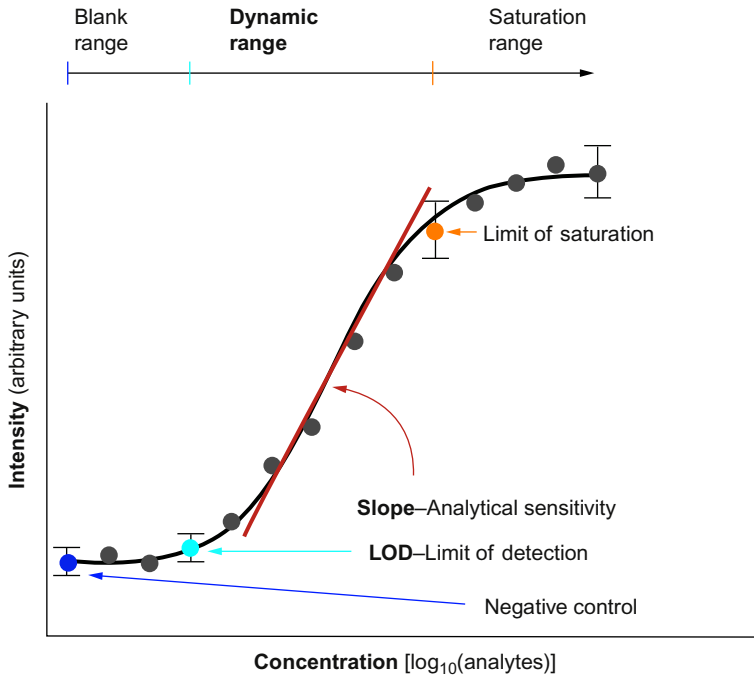


**Fig. 2** Visualization of the key assay metrics that is gleaned from a standard curve, which is a regression fitted to the assay output as a function of analyte concentration on a logarithmic scale. Within the dynamic range, the standard curve provides a quantitative relationship between the assay output and analyte concentration.

negative control and of the samples (Borysiak et al., 2016; Holstein, Griffin, et al., 2015). In accordance with international standards, the analytical sensitivity is the slope within the linear portion of the logistic fit and is not synonymous with the limit of detection (Borysiak et al., 2016). Beyond these metrics, the limit of saturation (highest quantifiable concentration or upper limit of detection) and dynamic range (range spanned by the lower and upper limits of detection) are also useful parameters with which assays are characterized (Fig. 2). While the limit of detection is typically reported as the amount of analyte present in the sample volume added to the device, in units such as femtomoles, rather than as an ambiguous concentration, the dynamic range is presented as the unitless number of orders of magnitude spanned, which is expressed as a base-10 logarithm (e.g., "three orders of magnitude").

## 4. BINDING KINETICS IN POROUS NETWORKS

One of the key parameters that influences the overall analytical sensitivity of a lateral flow assay is the affinity of the binding element to the target analyte (Karlsson, Michaelsson, & Mattsson, 1991). Such affinity, and the corresponding binding kinetics, is dictated by the summation of the interactions between a protein and the ligand to which it binds. These binding interactions consist of a number of molecular forces, including charge–charge interactions, hydrogen bonds, hydrophobic interactions, and van der Waals forces. Note that these forces will usually be modified by the fact that the binding element is immobilized on a matrix that has its own contribution to the forces felt by the ligand. In this section, we will consider the role of binding kinetics in assay sensitivity, with special consideration of the influence of reagent delivery.

The underlying kinetics of a binding reaction can provide insight about the interaction between an analyte and a ligand. Interpretation of a binding event can be directed by an understanding of the underlying kinetics of a reaction between an analyte and a ligand. A simple binding reaction involves an analyte (A) and a ligand (L) which reversibly forms a bound complex (AL). As shown in Eq. (1), the rate of association for the protein and ligand to form a complex is defined as $k_{on}$, and the rate at which the reaction reverses is defined as $k_{off}$. Three factors that should be taken into consideration are (1) the concentrations of protein and ligand that are present, (2) the concentration of complex that is formed, and (3) how quickly the complex breaks apart (Williams & Daviter, 2013). These three factors enable quantitative interpretation of a binding event within a Michaelis–Menten kinetics framework (Michaelis & Menten, 1913).

$$\mathrm{A} + \mathrm{L} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{AL} \tag{1}$$

The dissociation constant ($K_D$) of a reaction is defined as the rate at which the binding reverses ($k_{off}$) divided by the rate at which the complex associates ($k_{on}$). With these rate constants, concentration does play a role in the amount of complex that is formed since the observed net rate of formation (or dissociation), $k_{obs} = k_{on}\,[\text{analyte}] - k_{off}$. As the local concentration of a protein or analyte is increased, the probability of a collision between the two molecules, and subsequent binding event, also increases. When considering binding at a test line, the local concentration in the microenvironment of

test line influences the observed on rate for this interaction. Therefore, the transport of a protein to the test line through either convection or diffusion influences the rate at which a binding event can take place.

While traditional methods to measure reaction kinetics are able to provide an estimate for the kinetics of a binding interaction, the rate constants determined by kinetic measurement systems might not necessarily translate directly into those relevant in paper microfluidic systems. This is primarily due to two properties of lateral flow assays: the constant movement of fluid by capillary action and porous nature of the membrane. There are additional effects from the matrix itself, effects that can be seen whether or not a surface has been passivated by a blocking protein such as BSA. Porosity is a measure of the empty space in a material and serves as a key parameter in the design of the lateral flow assays (Ahmad, Low, Shukor, & Ismail, 2008). The larger the void space, or mean pore size, the smaller the surface area-to-volume ratio is for a given segment of membrane. When a ligand is immobilized onto the exposed surface area of a test region, larger amounts of protein can be deposited on membranes with smaller pore sizes. However, as pore size decreases, overall assay speed can suffer as the flow rate is reduced. While this may increase the probability of a collision event as the distance to diffuse to the surface decreases, the primary tradeoff is an increase in time from sample to result.

Experimentally determined rate constants provide insight into the affinity between a protein and a ligand that can translate to improved performance of an assay integrated into a paper microfluidic system. Specifically, empirically determined association and dissociation rate constants may shed light on the kinetics of assay binding for varying reagent delivery formats in porous media (Liang et al., 2016). Reagent delivery can be either sequential, in which each reagent is delivered independently across the test line, or premixed, in which the analyte and detection stack are mixed before they are delivered to the test region. The Fu group has demonstrated a 4- to 10-fold improvement in the limit of detection for the malaria antigen *Pf*HRP2 using the sequential delivery format (Liang et al., 2016). In contrast, recent work has also shown an improved limit of detection for the influenza nucleoprotein using the premixed delivery format (Abe & Yager, 2016). While the underlying mechanisms are not yet fully understood, the difference in performance of these two assays is likely due to differences in antigen binding; this could include multivalency of the antigen, kinetics of binding of the antibodies, or the size of the bound analyte–ligand complex. This body of work highlights that overall assay

performance is influenced not just by the affinity of the binding elements, but also by the manner in which they are delivered across the test region. As we discuss the available mechanisms for protein binding in the next section, these concepts regarding assay kinetics must be considered in the context of each assay.

## 5. MECHANISMS FOR PROTEIN BINDING

The analytical sensitivity of a lateral flow assay is first and foremost limited by the affinity of the binding elements for the target analyte (Karlsson et al., 1991). As mentioned in the previous section, the sensitivity and specificity of protein–ligand binding is driven by the binding kinetics of the interaction. A sandwich assay requires a minimum of two specific binding events: capture of the analyte onto a solid surface by a first ligand immobilized thereon and detection of the analyte with a label conjugated to a second ligand. Capture and detection of an analyte can be through an antibody, a designed affinity protein, or an aptamer, among others. While traditional lateral flow assays utilize antibodies as the capture and detection elements of a sandwich immunoassay, antibodies cannot be applied to all diagnostic targets. In porous media, additional considerations must be taken into account during assay design regarding the strength of immobilization and ability to flow through a porous network for capture and detection. In this section, we will present extant mechanisms for sensitive, specific analyte binding, including recent work demonstrating the use of designed proteins and nucleic acids as binding elements for protein detection.

### 5.1 Antibodies

Antibodies are the most commonly utilized binding element for lateral flow immunoassays, building from their use in immunoblotting. As the workhorse of the adaptive immune system, antibodies are developed by a host to bind specifically to an antigen (Lipman, Jackson, Trudel, & Weis-Garcia, 2005). Polyclonal and monoclonal antibodies can be raised in a live animal, or bacterial or eukaryotic cells, respectively, and then purified using affinity chromatography (Jayasena, 1999). Polyclonal antibodies are generated by the total antibody response for a given analyte, while monoclonal antibodies are specific to one epitope of interest. Monoclonal and polyclonal antibodies can have different diagnostic applications due to their differences in specificity and production, therefore, their use depends on the assay and

the analyte of interest. In both cases, each binding arm ($F_{ab}$) of an antibody serves as an epitope for binding, while the $F_c$ region of the antibody has biological effector functions that can also be utilized. Integration of antibodies into lateral flow assays is facilitated by the fact that most antibodies adsorb robustly to nitrocellulose, such as in Western blotting applications (Holstein, Chevalier, et al., 2015; Renart, Reiser, & Stark, 1979).

However, antibodies are limited in their diagnostic applications by a variety of factors. The most prominent of these factors is that production of antibodies is typically both labor intensive and expensive (Jayasena, 1999). Challenges include a lack of stability under extreme pH and temperature, batch-to-batch variability, limited control over functional sites, and the inability to target nonimmunogenic or toxic analytes (Mairal et al., 2008; Marx, 2013; Vermeer & Norde, 2000). A number of these limitations have been addressed through the production of antibody fragments that contain just the single chain or multivalent fragments (Binz, Amstutz, & Plückthun, 2005). These antibody fragments can be grown in bacteria, and therefore are much less expensive to manufacture and have greater specificity control. However, this only applies to targets for which antibodies can be produced in the first place, and not all antibodies can be broken down into smaller pieces, and remains stable.

## 5.2 Proteins

Affinity proteins are a class of proteins that have been utilized for the development of assays for the detection of target analytes. These proteins are expressed recombinantly in bacterial cells, enabling production that is both much simpler and lower cost than for monoclonal antibodies. One approach to develop proteins that bind to a target of interest is computational (de novo) design, in which the tertiary structure of a protein is predicted based on primary amino acid sequence (Whitehead et al., 2012). This design concept, also known as rational molecular design, utilizes molecular approximations that generally require further affinity maturation that enables improvement in binding affinity. Another mechanism for the development of binding proteins is phage display, which is instead based on evolutionary molecular design (Alarie, Sepaniak, & Vo-Dinh, 1990; Binz & Plückthun, 2005). Phage display typically utilizes a randomized library that is made through any of a variety of mutation techniques to create protein variants that bind to the target of interest and is expressed on the exterior of the phage; tight binders are identified by a simple filtration, and the binding

protein(s) are then amplified from the nucleic acid encoding it in the phage itself. Affinity maturation is also used with phage display to develop proteins with successively higher affinities.

One of the advantages of affinity proteins is that they can be designed to bind a specific epitope on a target protein, providing more control in the development of a sandwich assay. As these proteins can be expressed in bacteria, they can be produced rapidly and inexpensively. Designed proteins can also be expressed with additional functionalities, such as a binding site for conjugation to a fluorescent label or a paper-binding protein (Liu et al., 2014). Despite these benefits, proteins generated using de novo design require the existence of a high-resolution structure for the analyte of interest. As these crystal structures do not account for movement, and may represent a minority structure present in solution, predictions of proteins that will bind to a specific epitope can often be incorrect. Additionally, there are often some conformations of an analyte for which protein prediction is additionally difficult. One example of this is the hemagglutinin (HA) protein on the influenza virus. In the California/09 strain of H1N1, there is a mutation in the sialic acid binding pocket which makes it difficult to design a protein that can effectively bind in that pocket. While evolutionary design more closely replicates the mutations that take place in antibody production in vivo, it is limited in the degree of mutation that can be made to a single protein without significantly altering the overall structure, function, and stability of the protein (Paschke, 2006). In the case of both rational and evolutionary design, the developed proteins can be evolved to have specific desired properties, such as stability under specific or varying pH or temperature conditions (Fleishman et al., 2011; Heyd et al., 2003).

## 5.3 Aptamers

Another class of binding moiety that has been utilized in place of antibodies is aptamers. Aptamers are oligonucleotides (DNA or RNA) that can fold into well-defined secondary, tertiary, and quaternary structures (Chen & Yang, 2015). They are easily amplified by polymerase chain reaction, which can be done in a variety of conditions that are not restricted physiologically (Chen & Yang, 2015; Famulok, Mayer, & Blind, 2000). Manufacturing of aptamers that bind to a particular target is accomplished through a process known as systematic evolution of ligands by exponential enrichment (SELEX). SELEX screens large combinatorial libraries of oligonucleotides; they are then further refined using iterations of in vitro selection and

amplification (Ellington & Szostak, 1990; O'Sullivan, 2002). Higher affinity can be selected through additional rounds of enrichment. This process is similar to the production of proteins through the evolutionary design process.

Aptamers retain many of the benefits of designed affinity proteins, which include ease of production, low cost, and improved stability. They can be produced to bind any target, even ones that are nonimmunogenic or toxic. Because aptamers are nucleic acids, they have the ability to return to their tertiary structure after being unfolded by heat or pH (Lönne et al., 2015). This property is beneficial in the context of point-of-care diagnostics that need to have a long shelf life under variable temperature and humidity. Aptamers, like affinity proteins, can be designed with a single reactive group for site-specific conjugation to facilitate assay development (Chen & Yang, 2015). To this day, aptamers have not been successfully demonstrated for as wide a range of targets as antibodies. This is in part because polynucleotides do not possess the full range of chemical moieties as are presented by amino acid side chains.

Each of the molecule families listed earlier for binding an analyte of interest has its respective benefits and drawbacks. It is important to note that, in terms of assay performance, all three have been shown in the literature to have comparable analytical sensitivities in the low nanomolar to picomolar range (Chen & Yang, 2015; Gold, 1995; Holstein, 2015). For example, they have been used for the detection of the recombinant HA with varying degrees of success (Holstein, 2015; Holstein, Chevalier, et al., 2015; Shiratori et al., 2014; Woo, Lee, Yim, & Jeong, 2015). Fig. 3 is a graphical representation of all of these binding strategies for simultaneous capture and detection of the analyte of interest.

A critical requirement for the capture molecule in a lateral flow assay is that it effectively immobilized onto the porous membrane. Direct adsorption from a buffer is the technique most commonly used for paper-based assay development, though more specific techniques including covalent attachment and anchor-based immobilization can also be used (Holstein, Chevalier, et al., 2015). Careful consideration of the strength of the interaction and the overall membrane capacity must be taken when selecting a capture molecule for a paper-based assay. It has been proposed that protein adsorption to nitrocellulose is influenced primarily by electrostatic interactions, hydrogen bonding, and the hydrophobic effect (Přistoupil, Kramlová, & Štěrbíková, 1969). Accordingly, effective adsorption of a capture molecule is a critical parameter that must be

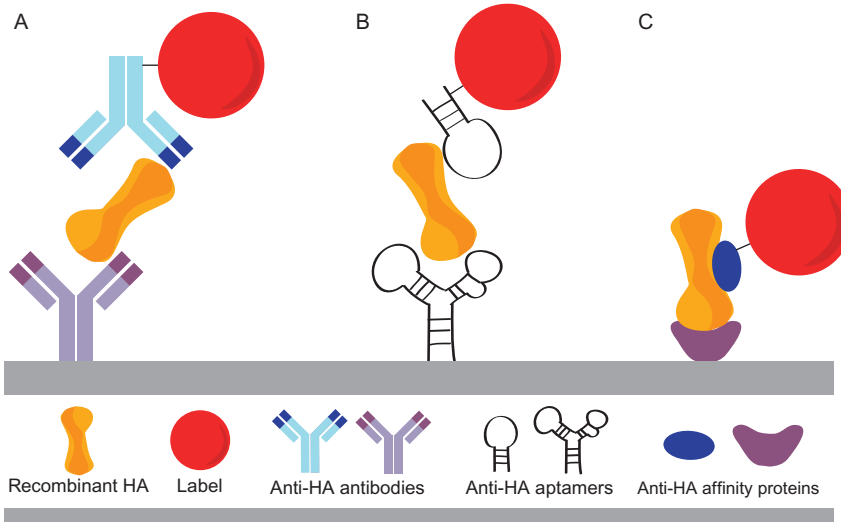


**Fig. 3** Graphical representation of three mechanisms that can be used for the capture and detection of recombinant hemagglutinin. In all three cases, the capture and detection elements were raised or designed to target different epitopes on the HA protein. (A) Antibody detection of recombinant hemagglutinin (Holstein, 2015). (B) Aptamer capture and detection (Shiratori et al., 2014; Wang, Zhao, et al., 2013; Woo et al., 2015). (C) Affinity protein detection (Holstein, 2015; Holstein, Chevalier, et al., 2015).

considered when selecting a capture element, whether antibody, aptamer, or affinity protein.

## 6. OPTICAL METHODS IN PAPER

Beyond the biochemistry underlying an assay, the choice of a detection label and the physics of detecting it will strongly impact the analytical sensitivity and limit of detection of a lateral flow assay. While conductimetric or amperometric readout of assays has been demonstrated, optical labeling is more commonly used, especially in commercial assays such as in pregnancy or HIV tests. Potential optical labels may be classified broadly as color-based, chromophoric labels, or as luminescent, photon-emitting labels. This section will elucidate the relative merits of potential optical labels, for which representative demonstrations to detect protein targets in paper-based assays are documented in Table 1. We will then discuss potential optical readers and image-processing strategies that are conducive to point-of-care protein detection.

**Table 1** Representative Performance of Optical Detection Methods to Detect Analytes of Interest

| Detection Principle | Potential Labels | Potential Readout Methods | Representative Limit of Detection | Dynamic Range ($log_{10}$) | References |
|---|---|---|---|---|---|
| Optical (absorbance) | Gold nanoparticles (AuNP), colored latex beads | Visual, scanner, smartphone, photodiode | 6.9 fmol | 4 | Holstein (2015) and Holstein, Griffin, et al. (2015) |
| Optical (chromogenic) | HRP-DAB; HRP-TMB | Visual, scanner, smartphone, photodiode | 0.06 fmol; 8.3 fmol | 1; 4 | Grant et al. (2016) and Stevens (2010) |
| Fluorescence | Organic fluorophores (e.g., Texas Red), quantum dots, lanthanide chelates | Visual, smartphone, photodiode | 0.86 fmol | 3 | Shah, Abe, Kauffman, and Yager (2016) |
| Phosphorescence | Lanthanide chelates | Visual, smartphone, photodiode | 0.35 fmol | 3 | Paterson et al. (2014) |
| Chemiluminescence | HRP-Femtoglow | Smartphone, photodiode | 0.51 fmol | 2 | Chen et al. (2016) |
| Thermal | Gold nanoparticles | Infrared (thermal) camera | Est. 0.2 fmol (32× w.r.t. AuNP) | 3.5 | Qin et al. (2012) |

## 6.1 Absorbance-Based Labels

Chromophoric optical labels are commonly utilized in lateral flow assays. Typical chromophores include low-cost, highly stable gold nanoparticles, which selectively absorb light at particular wavelengths (Hu et al., 2013), thereby enabling ready discernment of red lines against the uniform white background of the nitrocellulose membrane typically used in lateral flow assays. As a result, most commercial assays use chromophoric labels, including those for human chorionic gonadotropin and HIV antibodies. However, these labels have the drawback of large particle size (20 nm or larger), which contributes to a potential for aggregation under extreme salt or pH concentrations and to nonspecific binding to the nitrocellulose membrane. Both of these limitations may contribute to reduced signal-to-noise ratio, lower analytical sensitivity, and poorer limits of detection.

Chromogenic optical labels were adapted to lateral flow assays to address some of the limitations of traditional chromophores. As denoted by the term, chromogenic labels operate on the principle of producing a color change to denote the presence of analyte. Such labels were adapted from histological labels, including horseradish peroxidase (HRP)-catalyzed oxidation of diaminobenzidine (DAB) or 3,3′-5,5-tetramethylbenzidine (TMB) into a dark precipitate in the presence of the cosubstrate hydrogen peroxide. The speed and specificity of this reaction results in a marked reduction in nonspecific signal and improved signal-to-noise ratio compared to gold-based chromophores (Grant et al., 2016). Unfortunately, the reagent concentrations must be controlled fairly precisely; hydrogen peroxide must be produced at the time of use due to its instability (Grant et al., 2016), and the additional user steps or complexity are necessitated due to the additional reagents (Grant et al., 2016; Stevens, 2010); and the HRP enzyme itself is deactivated by hydrogen peroxide (Sennepin et al., 2009), all of which contribute to a relative dearth of literature utilizing such optical labels. Despite these potential drawbacks, we and others have demonstrated the suitability of chromogenic labels using the HRP enzyme for either wide dynamic range or low limit of detection measurements (Grant et al., 2016; Stevens, 2010).

## 6.2 Luminescent Labels

In contrast to absorbance-based optical labels, luminescent labels have the potential to improve the analytical sensitivity of lateral flow assays without a commensurate increase in assay complexity. Potential options include

fluorescent, phosphorescent, or chemiluminescent labels, all of which have been used to improve the signal-to-noise ratio because even when their signals are weak, the very low background at the wavelengths of interest enables the signal to be detected, but at the cost of requiring sensitive light detectors (O'Farrell et al., 2009, p. 88).

Fluorescent labels include organic fluorophores such as Texas Red or fluorescein derivatives, quantum dots, and lanthanide chelates, among others. Each of these absorb photons at particular wavelengths and reemit them at primarily longer ones as visualized by the labels' excitation and emission spectra, respectively. The difference in the excitation and emission spectral maxima is defined as the Stokes' shift, and larger Stokes' shifts are preferred due to ease of wavelength discrimination, simpler optics, and potentially improved cost and sensitivity (Lee, Nordman, Johnson, & Oldham, 2013; Li et al., 2010; Shah et al., 2016; Venkatraman & Steckl, 2015). Assay developers choose the wavelengths of interest of the excitation source and detectors to minimize cross talk, typically by using lasers or light-emitting diodes as the excitation source and by using optical filters on both the excitation source and the detector. This wavelength discrimination inherently improves the analytical sensitivities and limits of detection of fluorescence-based lateral flow assays compared to their absorbance-based counterparts. However, most organic fluorophores suffer from small Stokes' shifts and tend to photobleach, while quantum dots suffer from the same size considerations as chromophoric nanoparticles, and europium chelates must be excited in the ultraviolet spectrum below 350 nm (Wang, Nchimi Nono, et al., 2013). Moreover, many nitrocellulose membranes used in lateral flow assays are themselves fluorescent, which produces background luminescence that may also contribute to reduced signal-to-noise ratios (Walter et al., 2010).

Phosphorescent labels may improve the signal-to-noise ratio of lateral flow assays by enabling temporal separation of the excitation and emission of luminescent labels, and also by having larger Stokes shifts than many fluorescent compounds. In this optical detection scheme, fluorophores are deliberately excited into the nonfluorescent, phosphorescent triplet state, which can increase the time constant of photon emission by as much as a dozen orders of magnitude, thereby providing a means to visualize photon-emitting labels against a dark, nonluminescent background after the excitation source is toggled off. However, use of phosphors as labels in paper-based assays has been limited, few potential labels have been identified, and their use requires a specialized reader (Paterson et al., 2014).

Chemiluminescent (and bioluminescent) optical labels are akin to their chromogenic counterparts in that a photon-emitting substrate is produced by the catalytic activity of an enzyme, e.g., HRP (Ge, Wang, Song, Ge, & Yu, 2012), luciferase (Urata, Iwata, Noda, Murakami, & Kuroda, 2009), or alkaline phosphatase (Yu et al., 2015). The primary benefit of this method is that no illumination source is needed, eliminating the primary source of background noise for fluorescence and phosphorescence detection, and thereby greatly enhancing the sensitivity compared to chromophoric labels. Chemiluminescence has been incorporated into highly sensitive commercial enzyme-linked immunosorbent assays (ELISA) instruments. However, the drawbacks associated with chromogenic labels using the HRP enzyme are likewise applicable to chemiluminescent and bioluminescent labels, along with an added drawback of potentially requiring a specialized reader to image the lateral flow strips in a dark environment (Chen et al., 2016).

## 6.3 Optical Readout and Image Processing

The choice of optical label guides the selection of an appropriate reader to quantify the assay output. Potential readout methods include visual discernment by human eye, imaging with a scanner, photography via a smartphone or portable camera, or an electrical output with a photodiode. The choice of readout method will have ramifications on the assay reproducibility, analytical sensitivity, usability, and feasibility for point-of-care use.

Visual readout by human eye is by far the simplest readout method as it may enable design of an electricity-free assay and enable point-of-care use in austere conditions. Typically, an end user will discern the presence or absence of color (for a qualitative assay) or ascertain the intensity of a color (in a semiquantitative assay) to determine the relative concentration of analyte present. An alternate approach to render paper-based assays semiquantitative is to have spots that selectively change color if the analyte concentration is above a threshold, thereby enabling an end user to count the number of colored spots to ascertain a specific range of analyte concentration (Lathwal & Sikes, 2016a). However, the assay's resolution of analyte concentration is inherently quantized using this approach, and there is a potential that the results of visual readout may not be reproducible between users due to differences in eyesight. While these limitations are relatively inconsequential for qualitative assays including those commercialized for

pregnancy, the limits of visual readout may render it impractical for other protein assays that need to quantify analyte concentration.

Therefore, many assay developers now use electronic readout methods that enable high-resolution quantification of analyte independent of visual readout bias (Martinez et al., 2010), including stationary flatbed scanners and portable smartphones. The reader of choice during early stages of assay development, especially in our lab, tends to be a flatbed scanner connected to a computer, which enables taking images with high spatial resolution (Göröcs et al., 2014), with high bit depth, and most importantly, in a linear color space. A developer usually quantifies the mean or maximum pixel intensity of a region of interest corresponding to the analyte capture region, subtracts that of a local background region on the lateral flow strip to account for nonspecific binding, and corrects for nonuniform lighting by dividing by a measure of the illuminance, often a local background region adjacent to the capture region not on the lateral flow strip (Holstein, Griffin, et al., 2015). This analysis is typically performed in ImageJ, Adobe Photoshop, or MATLAB, among other programs. A standard curve is then fitted to the data, where $y$ is the normalized signal intensity and $x$ is the analyte concentration, usually using a four-parameter logistic curve as in Eq. (2) (Holstein, 2015):

$$y = d + \frac{a - d}{\left(1 + \frac{\log_{10}(x+2)}{c}\right)^{b}} \tag{2}$$

However, the results from this approach may lead to an unintentional bias in the results due to use of the sRGB color space. The sRGB color space is the current standard for computer and smartphone displays and digital photos. Unfortunately, the sRGB color space is nonlinear; gamma varies from 1 to 2.4 depending on luminosity (sRGB, 1999), which fundamentally prevents device-independent, accurate analyte quantification since the statistical assumptions underlying the generation of a standard curve often assume normally distributed residuals. There are three main methods to address this concern: incorporate the nonlinearity of gamma in the standard curve generation process, linearize the color data, or use a file format with a constant gamma. The latter option is usually the simplest, which leads many to use the TIFF file format in a linear RGB colors pace in lieu of more common JPG files in a sRGB color space, or to use the device-independent Lab

color space (Abe, Suzuki, & Citterio, 2008; Lathwal & Sikes, 2016b; Stevens, 2010).

These issues are also important when considering an assay with a smartphone or digital camera readout. Smartphones are highly portable and are conducive to the development of custom apps for assay quantification. Smartphones are, therefore, amenable to point-of-care use (Martinez et al., 2008), unlike scanners that require stable electricity, a computer, and highly trained users. However, it is important to consider the ramifications of rapid update cycles (typically annually), differences between smartphone models, eight-bit color channels, variations in ambient lighting, automatic postprocessing performed by the camera hardware, and unwillingness of end users to potentially contaminate their own device in a particular scenario when developing smartphone-based diagnostic assays.

For all electronic readout methods, the choice of detector will dictate the overall analytical sensitivity and limit of detection of the assay. These include the spectral sensitivity, quantum efficiency, field of view, and dark noise of the detector. While devices with broad spectral sensitivity and high quantum efficiency are advantageous, such systems suffer from poor dark noise, thereby reducing the signal-to-background ratio and offering minimal improvement to assay sensitivity. This tradeoff is an inherent limitation of jack-of-all-trades CMOS and CCD detectors that are found in modern cameras, smartphones, and scanners. In contrast, photodiode readout purports to improve assay sensitivity by enabling one to focus on the wavelengths of interest with minimal noise (since the noise is typically distributed over many wavelengths while the fluorophore emits at a more discrete wavelength) and by allowing signal integration over time, but at the expense of reduced field of view and lower spatial resolution. Their low cost, reproducibility, compactness, and ease of use render them highly suitable for point-of-care use (Ellerbee et al., 2009), as demonstrated by commercial devices such as the Qiagen ESEQuant or BD Veritor lateral flow assay readers.

## 7. SENSITIVITY ENHANCEMENTS (LOD)

Improving the sensitivity of a bioassay requires improving the signal-to-noise ratio of detecting the target analyte. This is accomplished by increasing the intensity of the signal of interest or by reducing the background intensity, both of which require optimizations either to the biochemistry mediating the assay binding or to the physics of the optical labeling.

## 7.1 Increasing Signal Intensity

Conceptually, the simplest way to improve the signal-to-noise ratio is to increase the magnitude of the signal intensity, the numerator. Potential options here include increasing the multiplicity of optical labels to targets via a signal amplification scheme or using optical labels that are intrinsically brighter. While increasing the intensity of the light source (to an extent) could conceivably increase the signal intensity (e.g., for fluorescence or absorbance-based measurements), the background noise may also increase commensurately (if the incident light intensity is increased beyond a critical level, perhaps due to Rayleigh or Raman scattering), which may not necessarily improve the signal-to-noise ratio.

Many labeling approaches conjugate labels to recognition moieties at a one-to-one or lower basis, wherein a single optical label binds on an average to one or more analyte molecules. One way to increase the signal intensity is to increase the number of optical labels bound to each analyte (Choi et al., 2010; Fu et al., 2010; Hu et al., 2013), either by selecting an appropriate linker that can bind to multiple epitopes of the analyte (Holstein, 2015), by designing the linker so that multiple optical labels may bind to the linker (Grant et al., 2016), or by coupling nucleic acid amplification to protein detection via immuno-polymerase chain reaction strategies (Janssen et al., 2013; Zhang, Li, & Le, 2012), among other approaches. For example, the HRP-DAB system should improve the signal-to-noise ratio compared to gold nanoparticle-based labels because a single HRP ligated to an analyte could produce hundreds or thousands of colored products; the signal could be increased even further by using the streptavidin–biotin linkage system due to the four binding sites that streptavidin (and avidin) has for biotin (Grant et al., 2016). In theory, the signal amplification strategy should increase the signal intensity by a factor up to the multiplicity of the label to analyte. In practice, however, amplification improves the analytical sensitivities and limits of detection of lateral flow assays by a factor less than the theoretical increase implied by the multiplicity, likely due to a commensurate increase in background intensity levels (Grant et al., 2016; Holstein, 2015; Janssen et al., 2013).

Modulating the incident light intensity with a greater magnitude entails both the selection of an appropriate, "bright" optical label and the selection of an appropriate wavelength at which measurements are made. For example, one novel readout method that may markedly enhance the analytical sensitivity and limit of detection of lateral flow assays is thermal readout

by measuring infrared emission from binding events at roughly 10 μm wavelengths in lieu of traditional visible light measurements in the submicron wavelength regime, which only measure absorbance of the bulk nanoparticle rather than detection of the binding event itself. The reader may consist of commercial smartphone thermal camera dongles, which could be used to image a standard gold nanoparticle-based lateral flow assay with no modifications to the bioassay itself. Proof-of-concept has been demonstrated by Qin et al, who have demonstrated a factor of 10 improvement in the limit of detection compared to visible wavelength imaging of gold nanoparticle-labeled assays (Qin et al., 2012).

## 7.2 Reducing Background Intensity

It is difficult to detect low levels of a signal of interest against high levels of a background signal. Among other factors, elevated background signal may be due to the choice of optical label or reader, to nonspecific binding of the detecting molecule or optical label, or due to the intrinsic brightness of the highly scattering paper substrate. Use of optical labels that are visualized against a highly luminescent background typically leads to a reduction in an assay's analytical sensitivity, as is the case for chromophoric and chromogenic labels against the typically white nitrocellulose membrane used in lateral flow assays. Since external lighting is needed for such assays, the reflected background white signal from the membrane overwhelms the optical reader, thereby reducing the signal-to-background ratio of the assay. In contrast, a luminescent optical label that operates in the dark and does not require external lighting may have an improved signal-to-background ratio, which in turn may improve the assay's analytical sensitivity and limit of detection (Venkatraman & Steckl, 2015).

Reducing nonspecific binding tends to be a more delicate process that involves optimizing the assay biochemistry following insights gained in ELISA. Since the nitrocellulose membrane used in lateral flow assays adsorbs proteins well and typically has a negative surface charge similar to an ELISA plate, there is a potential for the optical label or for the intermediary linker (detecting antibody, binder, or aptamer) to bind nonspecifically to the membrane (Holstein, 2015). Therefore, the lateral flow membrane is often blocked with proteins such as BSA (Lee, Kim, & Moon, 2013; Stevens et al., 2008), the isoelectric point of the intermediary linkers are optimized

(Holstein, 2015), and vigorous wash steps with an appropriate buffer (e.g., phosphate-buffered saline with added surfactant and blocking protein) are included in the assay procedure (Rohrman & Richards-Kortum, 2012; Shah et al., 2016; Stevens et al., 2008). In addition, the chemical bonds between the intermediary linker and optical label can be optimized themselves. The linker and label must be either covalently or noncovalently bound to each other, for instance via strong, noncovalent streptavidin–biotin interactions, among others (Hall, Chen, Chun, Du, & Zhao, 2016). Theoretically, noncovalently bound optical labels may be susceptible to dissociation in harsh conditions such as in the flow regimes present in lateral flow assays during wash or rinse steps, which may, in turn, reduce the signal-to-noise ratio. While a covalent bond between the linker and label is preferable due to its immutability ($k_{off} \sim 0$), noncovalent interactions are more commonly utilized since they too are conducive to modularly designed assays and interface well with commercially available reagents such as conjugated antibodies.

## 8. SUMMARY AND NEXT STEPS

Recent advances toward ultrasensitive protein detection at the point of care have demonstrated the possibility of improving the sample-to-result runtime of paper-based assays beyond that of laboratory-based ELISA while improving limits of detection compared to traditional gold nanoparticle-labeled lateral flow assays (Grant et al., 2016). Implementing strategies ranging from incorporating novel-binding mechanisms, optimizing reagent delivery formats, varying detection labels, and introducing washing steps have contributed to a two order of magnitude improvement in limits of detection as shown in Fig. 4. Unfortunately, these assays still suffer from requiring complex user steps and have poor limits of detection that are at least an order of magnitude worse than the most sensitive ELISA (Fig. 4). Despite these limitations, these assays offer the prospect of enabling rapid, sensitive detection at the point of care. Incorporation into autonomous detection platforms, such as those recently demonstrated for nucleic acids (Lafleur et al., 2016) or synthetic biology (Stein & Alexandrov, 2014), would fill that gap. Such a design may encourage the development of a new generation of rapid, ultrasensitive, and point-of-care protein assays, which could herald a breakthrough in patient care in both developed and developing countries.

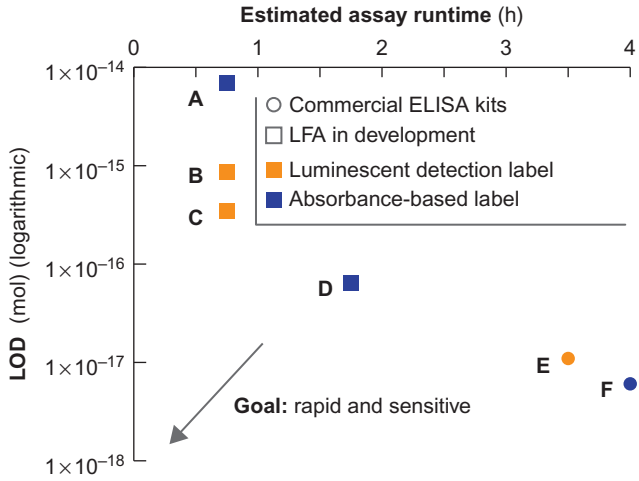


**Fig. 4** Visualization of the tradeoffs between the limit of detection (LOD) and estimated assay sample-to-result time for select protein detection mechanisms. Optical labels include (A) gold nanoparticles (Holstein, Griffin, et al., 2015), (B) fluorescent quantum dots (Shah et al., 2016), (C) phosphorescent nanoparticles (Paterson et al., 2014), and (D) horseradish peroxidase-diaminobenzidine (Grant et al., 2016). Extant point-of-care lateral flow assays have limits of detection that are at least one order of magnitude worse than those of the most sensitive commercial ELISA kits as reported in specification sheets (E and F) (ab188389—Free Prostate Specific Antigen (free PSA) Human SimpleStep ELISA® Kit., 2016; Guide to enzyme substrates for ELISA, n.d.).

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