Lateral Flow Immunoassay Systems: Evolution from the Current State of the Art to the Next Generation of Highly Sensitive, Quantitative Rapid Assays

Brendan O'Farrell (bofarrell@dcndx.com)

There is a traditional continuum in diagnostic technologies from highly accurate methods, requiring infrastructure and a centralized approach, to less accurate technologies that can be used in a decentralized or **point-of-care** (**POC**) testing strategy that requires little to no supporting infrastructure. **Lateral flow immunoassays** (**LFIAs**) have traditionally held pride of place at the lower end of that continuum.

Advances have allowed for the extension of performance of LFIAs into applications that require higher accuracy and sensitivity while still maintaining the advantages of the technology from the perspective of infrastructure requirements and user friendliness. This has allowed for improved application of this technology in decentralized testing environments, medical, and otherwise. Additionally, applications in the developed world have taken advantage of the improved performance in this assay format to move the LFIA format more in the direction of becoming a true laboratory-based system, utilizing the lateral flow strip as a component of a highly specified platform, with advanced facilitative technologies in the form of readers, specifically designed sample handling devices, and cartridge approaches with onboard functionality—essentially creating laboratory analyzers with the lateral flow format at their heart. Other applications for this technology fall in between these two extremes, demanding high performance and ease of use, coupled with mobile, facilitative technology for high-end field-based applications. In short, the LFIA, once the poor relation to laboratory-based tests, is being viewed more and more as a truly versatile technology, capable of more than adequate performance at all ends of the diagnostic continuum.

In order for this evolution in capability to occur, there has been a continuous improvement in materials, reagents, approaches to development, manufacturing equipment, manufacturing process technology, and the introduction of a whole new generation of facilitative technologies. The purpose of this chapter is to outline the basic principles of the lateral flow technology and to discuss the key elements of design, development, manufacturing, and supporting technology that allow for the use of this assay format in today's highly demanding applications.

Brief Overview of Lateral Flow – Current Market Scope and Standard Technology

THE LATERAL FLOW MARKET

The world market for lateral flow-based tests is estimated at US$ 3360 million in 2010 and, with a compounded annual growth rate (CAGR) of 7%, it will reach $4675 million in 2015 (Table 1).

This estimate includes lateral flow-based tests used in human and veterinary medicine, food and beverage manufacturing, pharmaceutical, medical biologics and personal care product manufacturing, environmental remediation, and water utilities. Lateral flow tests are also available and in development for biowarfare agents and pathogens such as anthrax, smallpox, avian influenza, and other potential biological weapons. These tests are not included in this market analysis because they are not in routine use at this time.

Worldwide there is a huge demand for rapid tests. In 2010, the US market accounts for US$ 1680 million (50%), the European market approximately 40% (US$ 1344 million), and the rest of the world the remaining 10% (US$ 336 million) of the worldwide market (Stratcom).

Over the past 30 years or so, lateral flow technologies have continued to evolve, until as of 2010, more than 100 companies worldwide are producing a wide range of tests with a total market valued at approximately US$ 3360 (Rosen and O'Farrell, 2011).

However with successive rounds of market consolidation and the emergence of Alere Inc. (Waltham, MA) as a major POC vendor, it is estimated that 27 companies hold 94% of the world market for POC tests in 2010. In 2005, 39 companies accounted for some 80% of the market.

The medical lateral flow test segment is generally seen as the most lucrative because worldwide there is a huge demand for decentralized availability of diagnostic tests. Lateral flow tests are well suited to replace laboratory-based immunoassays in decentralized POC testing locations.

The data presented here include all lateral flow-based POC immunoassays for self-testing that are sold

TABLE 1   World Lateral Flow Test Sales Revenue (US$ Million)

	2010	% Mkt	2015	% Mkt	CAGR %	No. of Companies
Clinical	2990	89	4100	89	7	At least 100
Veterinary	280	8	420	9	8	At least 50
Food and beverage	50	1	95	1	14	Approx. 20
Pharma/biologics	20	0.6	35	1	12	Approx. 20
Environment	10	0.2	12	0.2	4	At least 50
Water utilities	10	0.2	13	0.2	5	Approx. 20
	3360	**100**	**4675**	**100**	**7**	

Source: *Stratcom*, Montreal, Canada.

over-the-counter and those performed by healthcare professionals in hospital laboratories, hospital wards, clinics, community health centers, and physician offices. The data do not include the diabetes segment. These tests do not use lateral flow technology.

Clinical, lateral flow-based, rapid testing generated company revenues of US$ 2990 million and accounts for 89% of the total world market for lateral flow tests in 2010. With a CAGR of 7%, this segment will grow to $4100 million by 2015 (Rosen and O'Farrell, 2011).

ARCHITECTURE OF A STANDARD LATERAL FLOW ASSAY

A typical configuration for an LFIA is shown in Fig. 1. Traditionally designed assays are composed of a variety of materials, each serving one or more purposes, overlapping onto one another, mounted on a backing card using a pressure-sensitive adhesive.

The assay consists of several zones, typically constituted by individual segments of different materials, each of which will be briefly explained here. When a test is run, sample is added to the proximal end of the strip and to a **sample application pad**. Here, the sample is treated to make it compatible with the rest of the test. The treated sample migrates through this region to the **conjugate pad**.

In the conjugate pad, a particulate conjugate has been immobilized, typically a colloidal gold or a colored, fluorescent or paramagnetic monodisperse latex particle. This particle has been conjugated to one of the specific biological

components of the assay, either antigen or antibody, depending on the assay format. The sample remobilizes the dried conjugate, and analyte in the sample interacts with the conjugate as both migrate into the next section of the strip, which is the reaction matrix.

This reaction matrix is a porous membrane, onto which the other specific biological components of the assay have been immobilized. These are typically proteins, either antibody or antigen, which have been laid down in bands in specific areas of the membrane where they serve to capture the analyte and conjugate as they migrate by the capture lines.

Excess reagents move past the capture lines and are entrapped in the **wick** or absorbent pad. Results are interpreted on the reaction matrix as the presence or absence of lines of captured conjugate, read either by eye or using a reader.

Immunoassay formats are either immunometric (also known as sandwich or direct immunoassays) or competitive (or competitive inhibition), and the assay format can accommodate qualitative, semiquantitative, or in limited cases, fully quantitative assays. Direct assays are typically used when testing for larger analytes with multiple antigenic sites, such as hCG, Dengue antibody or antigen, or HIV. In this case, a positive result is indicated by the presence of a test line. Less than an excess of sample analyte is desired, so that some of the conjugated particles will not be captured at the capture line and will continue to flow toward the second line of immobilized antibodies, the control line. This control line typically comprises a species-specific anti-immunoglobulin antibody, specific for the
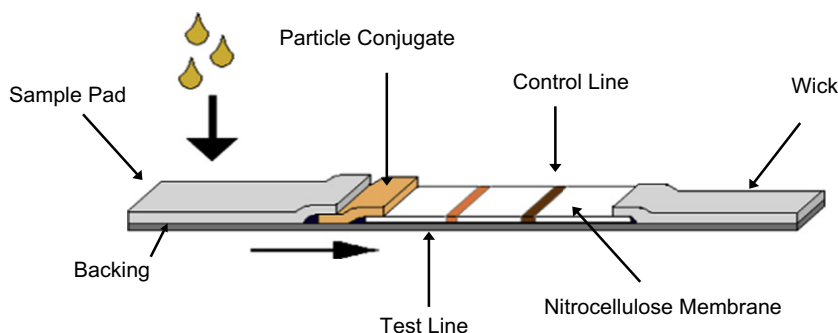

FIGURE 1   Typical lateral flow test strip configuration. (The color version of this figure may be viewed at www.immunoassayhandbook.com).

conjugate antibody on the conjugate. Competitive formats are typically used when testing for small molecules with single antigenic determinants, which cannot bind to two antibodies simultaneously. In this format, a positive result is indicated by the absence of a test line on the reaction matrix. A control line should still form, irrespective of the result on the test line.

MANUFACTURING PROCESSES AND METHODS

Figure 2 outlines a generalized manufacturing process for traditional lateral flow test strips. The materials and processes typically used for manufacture of each of these systems, and the ways in which the materials are used have remained largely unchanged for much of the history of LFIAs.

Processes and Equipment

Traditional and improved manufacturing technologies, equipment, and suppliers will be discussed.

The performance requirements for rapid assays vary considerably between market segments. In the most general terms, however, the performance of LFIAs has always been challenged in two major areas: reproducibility and sensitivity. The lack of strip-to-strip reproducibility has dogged the reputation of this assay format and prevented the adoption and application of these assays in many quantitative formats. Much of this lack of reproducibility comes from the materials used in the assays. However, the manufacturing tools and processes used also account for a considerable component of this inter-assay variation. The other performance element that is being driven to higher levels by market needs is sensitivity. This is a general trend in the diagnostic marketplace for all assay formats and in the context of considering manufacturing processes and tools, it must be remembered that rather than being a function of manufacturing process, the requirement for increased sensitivity is going to impose new requirements on the manufacturing process. Atypical processes such as lyophilization of conjugates into a bead and the utilization of nonstandard, nonparticulate reporter molecules, which in turn require new and controlled manufacturing techniques, are becoming more commonly applied in the effort to overcome variability and increase speed and sensitivity in lateral flow formats.

Traditional manufacturing process technologies: batch and reel-to-reel

The main processes required in the manufacture of an LFIA are as follows:

Dispensing

The addition of complex fluids containing a variety of compounds, including proteins, surfactants, and polymers in a controlled, reproducible way to the components of an LFIA is among the most technically challenging processes in the production of these assays. Two primary methods of reagent application to materials are used (Tisone and O'Farrell, 2009).

For the addition of bulk volumes of fluids, such as membrane blocking reagents, sample pad, and conjugate pad treatments, impregnation of the materials is performed by **dipping** into tanks followed by **blotting** and **drying**.

For the addition of quantitative volumes of fluids in a controlled manner, such as test and control line dispensing and conjugate deposition, more controlled, highly accurate, and reproducible processes are needed. The deposition of membrane test and control lines has been performed using a variety of dispensing methods, which in practice have been variably quantitative and equally variably practical and scalable on a manufacturing basis. What have emerged as broadly applied technologies in the industry today are basically three methods of dispensing quantitative volumes of reagents onto surfaces.

Contact tip dispensers The FrontLine gliding tip dispenser from BioDot Inc. is one example of a contact tip dispenser. Other producers of this type of dispensing method include Kinematic Automation and Imagene
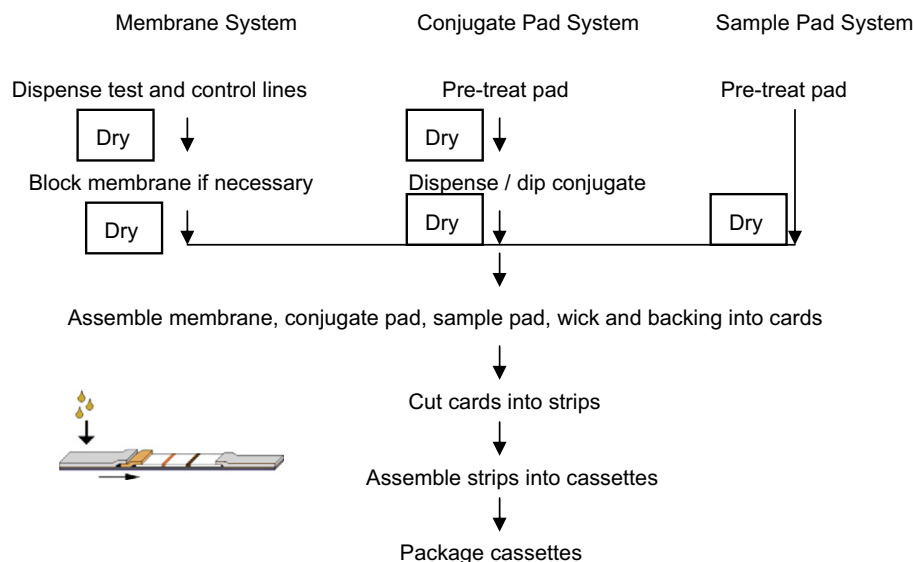

FIGURE 2 Outline of a typical lateral flow manufacturing process. (The color version of this figure may be viewed at www.immunoassayhandbook.com).

Technology. The dispenser consists of a syringe or other positive displacement pump with a flexible tip that drags across the surface of the membrane. The amount of fluid pushed through a contact tip system is quantitative, as defined by the accuracy of the pump used. However, the amount of fluid absorbed per unit length of the material is not necessarily either quantitative or quantifiable. This is because the absorption rate of the nitrocellulose material typically used is dependent on material-related issues (Tisone and O'Farrell, 2009). These include

- hydration of the membrane,
- the pore size of the membrane,
- membrane surface characteristics (smoothness, dust),
- fluid factors including viscosity and protein concentration,
- environmental factors such as the ambient relative humidity of the striping process area.

The variability that these factors introduce into a deposition process results in a variation in deposited volume per unit length and can result in variations in developed line width. This will in turn introduce potentially critical levels of variation into a quantitative assay system. Additionally, there is the possibility of damage to the materials, particularly membranes, caused by the dragging of a tip across the surface of the material. These dispensers tend to be very reliable, however, and require little maintenance beyond careful cleaning, so from the perspective of manufacturing, they can provide a good solution to the requirement for relatively accurate dispensing of test and control line reagents.

Noncontact, pump-driven solenoid dispensers These include BioDot's BioJet Quanti, which uses a drop actuator such as a solenoid hydraulically pumped with a positive displacement system such as a syringe pump. This method has the advantage of no contact between the material and the dispenser tip, improving the consistency of dispensing while minimizing the possibility of damage to the material surface caused by dragging a dispense tip across the surface of the material. These advantages are balanced by a requirement for careful treatment of the dispenser heads, prevention of clogging, degassing of fluids to get very accurate dispensing, representing a greater maintenance burden for a manufacturing process. The balance between the benefits of very accurate performance and the cost of implementation on a manufacturing scale must be evaluated.

Quantitative airbrush-type dispensers The deposition of particulate conjugates onto pads that have variable surface characteristics, such as glass fibers and polyester, is generally achieved using either an impregnation or a spraying process. Most particulate conjugates, such as 40 nm colloidal gold or 100–200 nm latex particles cannot be dispensed using solenoid valves or contact tip dispensers due to clogging or damage to the dispense head. AirJet-type dispensers are therefore commonly used. BioDot produces the AirJet Quanti, which is a unique technology for the quantitative deposition of fluids or particulates, either for impregnation or for production of fine lines or spots on a substrate. Another variant on this technology is produced by Kinematic Automation. This dispenser consists of a pneumatic spray generator hydraulically pumped with a

positive displacement system such as a syringe pump. The typical generator is a pneumatic driven aerosol similar to the artists' airbrush. In this case, the dispenser/generator provides a quantitative delivery of a spray in terms of microliters per second which, when synchronized with motion, can produce both individual spots and lines.

Impregnation

Impregnation is the saturation of the material with a reagent. This is usually followed by a tightly controlled drying process. This can be done by soaking sheets or webs of materials in a reagent or using a dispenser to saturate the material. Either process can be performed using sheets or webs in various formats. Typical applications of this method are the pretreatment of conjugate or sample pads with solutions of polymers, surfactants, and proteins to make them more hydrophilic, to control flow rates, or to impregnate them with sample treatment buffers. Blocking reagents are also typically applied to membranes using impregnation methods.

The basic process involves the immersion of the material, either in pad or roll format, in a vat of the fluid to be applied. Many of the materials used in these assays require a somewhat lengthy dwell time to ensure complete impregnation of the material with the fluid due to the extreme hydrophobicity of the materials (e.g., glass fiber or polyester pads). These processes may be either batch or continuous in nature. If they are batch, pads or sheets are immersed in the solution to be applied, agitated by hand or *via* another method such as ultrasonic vibration, to ensure even uptake of the fluid. They are then removed, blotted, or squeegeed to remove surface moisture and dried using lyophilization or forced air at high temperatures.

In a continuous process, the material, still in roll format, would be passed through the bath at carefully controlled speed and contact angles, then squeegeed and dried, usually in a drying tunnel or tower. This process of immersion and drying, particularly in batch format, is a major source of variation for a variety of reasons including the uneven uptake of fluids and inconsistent drying of the materials in batch ovens.

Drying

Drying is one of the most critical processes to achieving consistency, stability, and sensitivity in LFIAs. Multiple drying processes are typically performed during the production of a strip, with different aims in each case. For example, when test and control lines are dried onto membranes after dispensing, the aim is to end up with a stable coating of immunoreactive protein. As a result, the drying process is critical to the ultimate performance of the test and must be very carefully controlled. The degree of drying, and method and timing of drying, are critical to achieving an active, stable protein on the surface. Control and consistency are key, as the timing, method, and degree of drying of the protein can be critical to the refolding of the protein into an active conformation after drying.

In general terms, when a protein solution is dried down onto a membrane surface, the protein partitions to the surface as the moisture is removed from the system. An initial charge attraction brings the protein to the surface,

where after a hydrophobic binding process occurs. During this hydrophobic binding process, the protein exposes its hydrophobic regions to the membrane surface. Over time some refolding of the protein occurs, ideally resulting in an active conformation. This is all done in the presence of membrane wetting agents—surfactants—that can have effects on the process and the ultimate activity of the protein. The degree of drying, the speed of drying, and the method of drying can all be optimized for individual proteins in order to generate optimal activity after the drying process. Control of temperature, timings, and humidity levels therefore become critical factors in the efficacy and reproducibility of drying. The same degree of control is critical when drying particulate conjugates, such as colloidal gold or monodisperse latexes conjugated to proteins. Obtaining even drying, with the correct degree of moisture removal, is critical to achieving stability of the dried conjugate and facilitating the homogeneous rewetting of conjugate pads and efficient resuspension of the conjugate particles.

Two basic drying processes have been used in the lateral flow industry: forced air at high temperature and lyophilization. Lyophilization is inherently a batch process with low process throughput, and hence in spite of its superior drying properties, it is not often used for pads. The common approaches for forced air drying are batch ovens or in-line drying ovens for web formats. Having said that lyophilization is becoming more common in the production of alternative conjugate formats, such as pelletized conjugates that are stored in a sample collection device or onboard a cassette. If properly designed, conjugate pellets can be extremely stable, even when stored at relative humidity of 20% and can give extremely consistent and efficient release of conjugates into assay systems. This will be further discussed later in the chapter.

Slitting

In the context used here, slitting is a cutting process used on materials along the web axis of the laminate, while cutting denotes cutting perpendicular to the web axis to form individual test strips or card lengths of materials. Slitting is principally used to cut wide sheets or web stock of sample and conjugate pad materials into thinner webs for lamination. Materials can be pre-slit before treatment or after treatment, depending on the design of the overall production process and the characteristics of the material, including tensile strength and brittleness.

Lamination

The lamination process is based on the use of either strips or webs of the different materials bonded to a pressure-sensitive adhesive on a plastic backing surface. The plastic backing comes with a protective release liner, which is removed prior to lamination. Lamination is performed, typically, using semiautomated laminators from manufacturers such as BioDot and Kinematic Automation or using in-line equipment that laminates rolls of treated materials to rolls of backing material, resulting, typically, in a cutting process that creates card lengths of materials that are further processed to individual strips in subsequent steps.

Cutting

The cutting process is used to cut the final test strip from the laminate either for bottling or assembly into a plastic cassette. There are basically three types of cutters that are used:

- guillotine,
- single rotary blade,
- rotary card cutter.

The guillotine cutter cuts one part per cut cycle and can be used for high-speed cutting of laminates or for cutting individual test strips for a pick and place assembly operation. The rotary card cutter cuts a laminated card into test strips at high speeds using a series of rotary blades to simultaneously cut the full length of the laminate card into individual strips.

Cassette assembly

The typical test format is a test strip with a plastic housing, which can also include a desiccant. In this case, the test strip is either manually or automatically placed into the cassette bottom, and the top part is placed and either welded or snapped together with the bottom part. Additionally, there is a growing movement toward the inclusion of radio-frequency identification (RFID) tags in cassettes, which are critical to functionality in reader-based applications. These tags are typically programmed and inserted into the cassette at the time of bulk cassette assembly. Cassette assembly is typically a process bottleneck, requiring either high labor input if the process is manual or the use of complex, customized machinery to complete the assembly process at a reasonably high rate of parts per minute.

Packaging

This operation consists of packaging the cassette in a foil pouch with a desiccant, and in some cases, other materials associated with the test. Packaging can be done manually using preformed pouches where the operator places the cassette and other parts and then seals the pouch with a rotary sealer. This also can be done automatically where the pouch is formed in line with the other operations.

Process options

Two basic approaches to these processes are possible and commonly practiced, namely batch processes, which require a high degree of manual labor input, and in-line (or reel-to-reel) approaches, which minimize this manual labor component.

Batch manufacturing

The basic premise of the batch manufacturing technique is that individual cards of 150–300 mm in length (typically) are processed individually. This allows for the use of low-cost equipment but has the disadvantage of high manual labor input, poor process repeatability and often high product variability. This processing method is typically used for relatively low production volumes.

Batch processing is typically performed with a number of tabletop instruments that include

- an XY motion system with dispensers,
- impregnation tanks,
- drying ovens,

- manual or semiautomated laminator,
- cutters.

The XY motion system is typically equipped with at least two line dispensers for test and control lines and one conjugate dispenser.

The batch method is basically the same for both R&D and manufacturing and can be scaled to higher volume manufacturing by using duplicate systems. There are two major issues with the batch approach, the first being that quality is heavily influenced by manual operations and second being that the batch process cannot directly be scaled to in-line processing. The major advantages are lower capital cost and ease of use (Fig. 3).

In-line manufacturing

The alternative to batch processing is reel-to-reel or in-line processing. In this method, materials are processed as continuous rolls of 50–100 m in length through to the end of the lamination process. This results in better process control, higher throughput and lower manual input and product variability.

The in-line equipment generally consists of modules for reagent processing and drying, processed web slitting, and lamination of materials to a backing card.

The in-line reagent processing unit performs dispensing and impregnation applications and has capacity for in-line drying and automated QC. Different dispenser types can be mounted to this system, identical to those used in batch processing, including contact, noncontact, and spray dispensers. The unit can also accommodate a dip tank module, which consists of a reagent reservoir with a roller system, used to impregnate a moving web. Impregnated or dispensed upon webs of materials are dried using integrated drying tunnels or towers.

The function of the in-line lamination system is to laminate all of the materials that have been pretreated and slit to the appropriate widths to a plastic backing with pre-slit release liners (Fig. 4).

Final device assembly

The final assembly is generally the most operation intensive process in the production of the LFIA. The design and implementation of automation for this process are confounded by the fact that the plastic housing design is usually custom to the individual producer or product line. This results in the need for customized tooling for each housing design. Recent innovations in assembly methods, described below, have the potential to minimize the impact of this customization requirement, however. Assembly processes can be considered as being either batch (manual) or continuous (automated).

The batch mode of assembly is essentially manual, with associated tools for cutting, assembly, and packaging. The equipment needed includes cutters, tooling for closing the plastic housing, and a sealing machine with preformed foil pouches. The strips are cut using high-speed guillotine or rotary card cutters where the cut strips are collected in bulk. Operators then individually place cut strips into the bottom plastic housing, place the top, and use a pressure tool to snap the two parts together. An operator then places the part in a foil pouch with a desiccant and any ancillary components prior to sealing using a manually operated sealing mechanism. This approach is the least capital and most labor intensive. It is also the worst scenario from the point of view of quality, which has the potential to degrade with increasing volume and number of operators. In addition to being the most susceptible to operator error, this step in the process also involves the maximum number of part handling operations, which can both damage parts and imparts a level of variability due to operator-dependent errors.

For high-quality and/or quantitative test formats, it is generally important to implement as much automation as is practical and cost-effective for the assembly and packaging of the product. It is also critical that environmental controls be implemented, with relative humidity controlled at less than 20% at a comfortable room temperature of 20–25 °C. The least capital-intensive approach to implementing automation would be to use a workstation where laminate cards or roll stock is fed to a cutter, which places the cut strip in the plastic part. The cutter system should have bad mark sensors that recognize parts on the laminate that have been marked upstream. This part is cut and rejected at the cut operation. The plastic cassette parts are then hand placed and removed from the workstation. Once the test strip is protected by the plastic housing, the device is more amenable to manual handling.

The next level of automation is to implement full in-line or automated processing. In this approach, a machine is used that integrates all of the assembly operations, from cutting to placement of the strip, QC of the strip, placement of desiccants, placement and closure of lid, insertion into a pouch, and closure of the pouch. Printing, labeling, and RFID insertion can also be included in the line. This can be done at a rate that generates very high throughput. These machines tend to be highly customized and highly capital intensive, however.

The major equipment suppliers for this type of strip processing steps in the developed world are Kinematic Automation and BioDot Inc. Recently, there have been some entrants to the assembly market, such as JOT Automation (Finland), who are taking a modular, robotics-based approach to this type of assembly work. This approach has the potential to reduce the amount of customization required and may result in reduced capital equipment costs, particularly when multiple product lines are being assembled.

Other processes

Other critical manufacturing processes include molding of cassettes, assembly of cassettes, packaging of cassettes and buffers, and alternative processes, such as lyophilization, all of which are custom processes dependent on the design of the test. Ancillary processes that must also be performed include printing of labels and barcodes, insertion of RFID tags, labeling or printing of cassettes with logos, barcodes, and areas for patient identification, pouch sealing, box assembly, and packaging.

Summary

The basic premise from which we begin in the manufacture of LFIAs is that we are dealing with an inherently complex system with numerous sources of variation built into the product. Many of the manufacturing steps are designed and controlled with the aim of minimizing the contribution of the variation in materials and reagents to

Batch Dispenser: BioDot XYZ 3050

Batch Cutter: BioDot CM 4000

Batch Laminator: BioDot LM 5000

Batch Drying Oven

FIGURE 3 Typical batch manufacturing equipment. (The color version of this figure may be viewed at www.immunoassayhandbook.com).

overall product variability. Great care must be taken during the design and development process to ensure that the processes developed can be scaled and controlled appropriately in order to minimize the introduction of unnecessary sources of variability into product performance.

Evolving Future Performance in POC Diagnostics

This section describes some of the more relevant technical issues that affect the future of POC testing and rapid diagnostics. The goal is to discuss the *status quo*, certain key development approaches being taken to the improvement of performance in rapid tests for POC use in field and laboratory environments, and to discuss unmet needs in rapid medical diagnostics in particular.

One major goal of technology design for POC applications is to extend the reach of more accurate technologies to decentralized testing scenarios, where they can often be of most use. Developments in this regard are proceeding down multiple paths but can be broadly categorized into two areas: efforts at improving the performance of existing technologies, and more radical approaches to the development of new technologies in the form of lab-on-a-chip and specifically designed approaches to the challenges of molecular diagnostic system application in POC

environments. The focus of this chapter is on lateral flow; therefore, the majority of this discussion will be given over to the ways in which performance in lateral flow systems can be improved. Some mention will be made of the use of lateral flow technology as the detection component of molecular diagnostics later in this chapter, however.

IMPROVEMENTS IN EXISTING TECHNOLOGY

In order to improve the performance of the system such that it can be applied effectively at the low resource end of the application spectrum as well as in higher end applications in developed world environments, innovation has been required in each of the key components of the lateral flow system. The key component technologies that must be addressed in the rational development of a POC diagnostic device for any application are
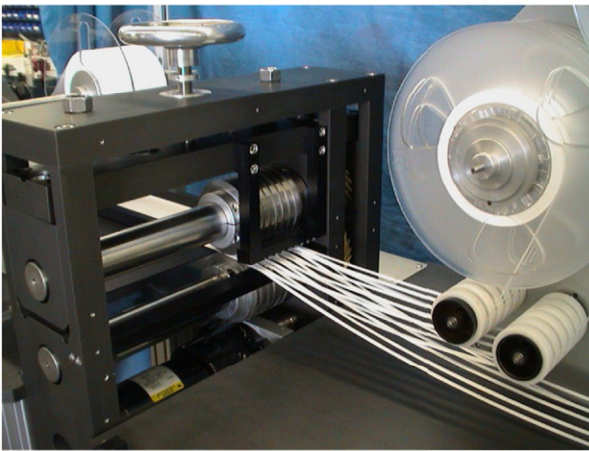
- sample collection and handling (e.g., concentration and preparation),
- recognition and signal generation technologies,
- readout and signal transduction technologies,
- device (cartridge) design.

Alongside the design of these systems, the design and implementation of carefully controlled manufacturing processes are critical to the ability to produce highly reproducible, quantitative, or multiplexed systems. Each of these system elements will be discussed with examples.

Reel-to-reel dispensing, dipping and drying equipment: BioDot RTR 4500

In-line material slitter (BioDot Inc)

Reel-to-reel lamination system: BioDot LM 5000

FIGURE 4 Typical in-line manufacturing equipment. (The color version of this figure may be viewed at www.immunoassayhandbook.com).

Sample Collection and Handling

For many applications, traditional lateral flow formats are capable of providing sufficient sensitivity. However, there is a growing demand for sensitivity in many infectious disease applications, for example, that approaches that of nucleic acid amplification and detection methods (LaBarre, 2011). Standard approaches to labeling and detection in lateral flow are unlikely to reach the required sensitivities

for these applications. Sample preparation is therefore the key to improved sensitivity and overall performance in many instances. It should be remembered that a large element of the appeal of LFIA and other point-of-need assay systems is that they should provide where possible a complete "sample-to-answer" solution in a single step. It is therefore critical to consider the system as a whole, including the sample, the sampling method, the sample pretreatment methodology, and the concentration of analyte in the system. Analyte concentration can be a confounding factor, either when it is too high or too low for detection, and sample treatment can and must be used to overcome related issues.

Sampling refers to the generation of a representative sample of an inhomogeneous object. This inhomogeneity presents a challenge to the success of the analytical method. As applied to highly sensitive rapid diagnostics, it is not the absolute sensitivity of the system that is the most critical factor, rather it is the ability to acquire as representative a sample as possible and that ultimately, it is the concentration of the analyte that one can detect in the primary sample that is critical. Sampling and pretreatment methods, primarily concentration and the removal of potential cross-reactive agents and reduction of background, are critical to determining the availability of many analytes for detection in an assay. Additionally, in certain circumstances, high concentrations of analyte can be a confounding factor in an immunoassay.

Sample handling and/or treatment can include multiple variations depending on the sample type, including plasma separation from fingerstick or venous whole blood, filtration, and breakup of mucins in saliva or respiratory samples or changes in pH of urine, to name a few examples.

In any assay system to be deployed in a decentralized testing environment, the sample collection, treatment, and delivery method must be simple, robust, foolproof, and ideally an integrated component of the test device. Minimal user-dependent steps should be required.

The specific example of whole blood treatment strategies for lateral flow will be considered in the remainder of this section.

Collection options for whole blood from fingerstick and separation of plasma for testing in lateral flow applications

The key considerations for the design of integrated testing solutions for whole blood design begin with the specification of whether the blood sample is to come from a tube, and if so what anticoagulants need to be considered, or whether the sample is to be taken from a fingerstick. The primary processing steps for the blood sample are

- metering of quantitative or semiquantitative samples of blood from the tube or fingerstick,
- separation of plasma from the sample without significant hemolysis,
- delivery of the sample, either neat, neat with a chase buffer, or pre-diluted, to the device.

The creation of solutions that achieve several or all of these steps in an intuitive, user-friendly way is a constant challenge, and the needs of every assay system are different.

Sample collection and transfer

If samples are to be drawn from a tube, the quantitative transfer to the device is made relatively simple through the provision of low-cost pipettes/droppers for use in field environments, or, if in a laboratory situation, it is assumed that quantitative pipettes are available for use. Some innovation is possible even in this area, however, with the use of low-cost, sharp-free customized devices for collection and delivery of fixed volumes from blood tubes. Such devices have been designed. One example is shown in Fig. 5 (Zeis 2010).

Depending on the strategy to be used for plasma separation and delivery to the device, fingerstick applications can also require innovation. Collection of fingerstick samples can be done in a variety of ways. Off-the-shelf capillaries are commonly used, as are low-cost, disposable semiquantitative pipettes. Some more novel approaches can be taken, however, including the use of capillaries with integrated dispense pipettes that can be readily customized for volume and reproducibility (Fig. 6). Collection using sponges is also possible, an approach that integrates with the use of vertical filtration options (see below).

Plasma separation and delivery to the test

Separation can be achieved in a variety of ways in rapid assays.

Filtration membranes Many lateral flow type systems utilize an in-line blood separation membrane, such as Pall Vivid™ or Cytosep™ membranes. These systems are relatively efficient, although they are limited in the volume of sample that they can handle. If they become overloaded, red blood cells are released into the assay, causing background issues. Often the system will require either a predilution of the sample or a chase/wash buffer to follow in order to wash the plasma clear of the membrane and to provide enough volume to wet the system completely (Fig. 7).

Other methods Another approach is to use active vertical separation, an approach that has been used in plasma separation devices for other areas of application, such as the Demecal® device shown in Fig. 8. This method is fast and user-friendly for POC applications. On the downside, the risk of hemolysis is greater than with other methods, however, careful optimization of the filter stack can yield the desired product characteristics for LFIA testing.

Recognition

The recognition and signal generation elements form the essential components of the analytical device in meeting high-performance demands, producing a signal that is in some way related to the presence and/or concentration of the analyte to be detected.

The recognition elements are the biological recognition systems of the assay, and in immunoassays, they are typically antibody–antigen based, although some applications call for other approaches including avidin–biotin or nucleic acid hybridization, for example. Careful screening and selection of reagents are absolutely critical to performance for these applications. Assay performance will be affected
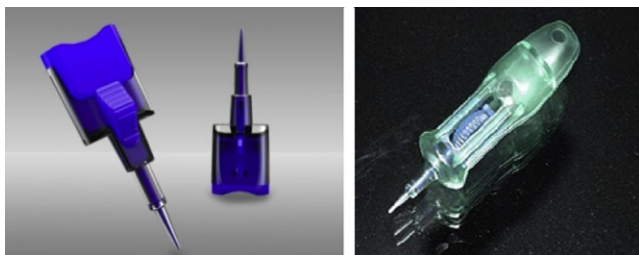

FIGURE 5 Sharps-free whole blood collection device. Graphics provided by Symbient Product Development. (The color version of this figure may be viewed at www.immunoassayhandbook.com).
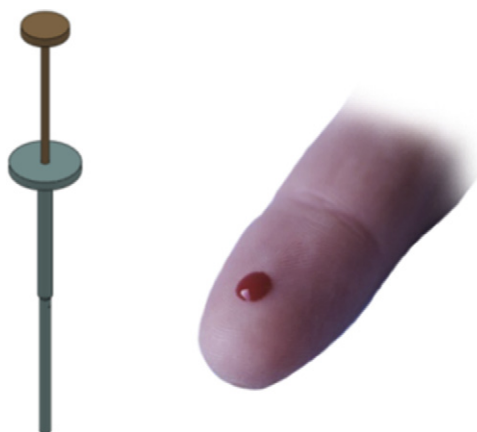

FIGURE 6 Capillary with integrated pipette. *Source: Symbient Product Development, CA, USA.* (The color version of this figure may be viewed at www.immunoassayhandbook.com).
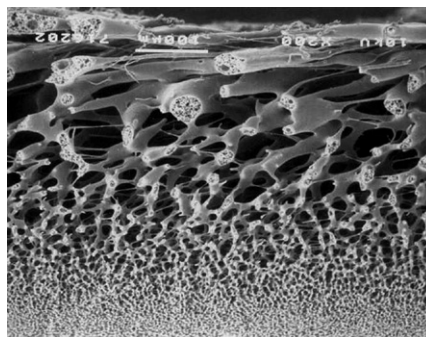

FIGURE 7 Red blood cell separation material, Vivid PSM (Pall Corporation).

FIGURE 8 Demecal blood collection kit (LabAnywhere, Haarlem, the Netherlands). (The color version of this figure may be viewed at www.immunoassayhandbook.com).

by variables such as antibody quality, including specificity and affinity, and also by parameters such as the binding rate constant and the antibody class. Different characteristics are required for high performance in an LFIA system than in an ELISA, for instance, due to the mechanics of the system.

Antibody selection for lateral flow assays

Overall rapid assay performance is affected by a number of variables including antibody quality. This includes such obvious parameters as antigen specificity and affinity (as defined by the dissociation constant). However, more important may be things like the binding rate constant, which is typically not measured during antibody screening and selection and antibody classes. Some subtypes are easier to conjugate or fragment and can pose special challenges with respect to purification, solubility, and long-term storage. IgG3, for example, is not bound very well by Protein A and tends to precipitate during freezing.

Key considerations involving antibodies in the lateral flow system include

- whether to use monoclonals or polyclonals,
- how to select and screen antibodies,
- how best to conjugate them,
- how to immobilize antibodies onto a membrane,
- how to optimize around the characteristics of the antibodies.

Monoclonal *vs* polyclonal

Monoclonal antibodies tend to be preferred over polyclonal for most systems. However, polyclonals can give adequate results in many cases. Commercial and antigen-dependent issues also come into play. Supplies of polyclonal antibodies are subject to variability unless a sufficient quantity can be obtained for the lifetime of the product in a single pool. Repeat bleeds from a single animal will be subject to marked variability over a period of time and if the animal dies the characteristics of antibody from another animal will be significantly different. The commercial issues are that the lifetime volume requirement is not predictable in advance, and validation of an alternative pool of polyclonal antibody is costly and may involve re-optimizing other elements of the LFIA to match the new pool. Many pools may need to be assessed to provide the closest match (Table 2).

The methods chosen for selecting and screening antibodies are important and will be discussed below.

The specific nature of the antibodies will have consequences with respect to conjugate preparation and immobilization on the membrane.

Unless the assay under development is for something present at concentrations higher than nanomolar, an antibody with high affinity is required. However, high "affinity" may not be sufficient. In the lateral flow format, this affinity needs to be driven primarily by a fast on-rate (k_{on}). (Note that this constant is also known as the association *rate* constant, k_a, but k_{on} is often used to avoid confusion between k_a and K_a, the association constant, which is also known as K_{eq}, the equilibrium constant.)

If we consider the kinetics of the LFIA, there is a test line zone that is typically in the order of 0.5–1.0 mm wide and a flow rate in the range of approximately 0.1–0.7 mm/s. This yields a potential time for binding at the test line of somewhere between 1 and 6 s in most systems (Brown, 2009). Hence, the need for a high on-rate in the antibodies.

The situation is a little more favorable for the conjugate, in that it spends more time in contact with the analyte. The "effective" binding reaction starts with resolubilization of the conjugate and ends after the conjugate passes the test line. This time is typically on the order of 10–20 s but can range up to many minutes for the last particles released from the conjugate pad. Note that for antibodies with a very slow on-rate, the first particles released (which usually represent the majority of particles) will impinge on the capture line having only a few copies of antigen bound, resulting in lower sensitivity than if the conjugate particles were saturated with analyte. This is one reason why premixing of sample and conjugate can be advantageous (more on that later).

Antibodies on conjugates

Colloidal gold concentrations are often expressed in terms of optical density or absorbance at 520 nm. A "1 OD" solution of 40 nm gold contains 990 billion particles/mL. The maximum amount of antibody that can be bound to a gold particle depends on the surface area of the particle. For gold colloids, this is about 150 IgG molecules per particle if 40 nm gold is being used (Table 3).

With gold, it is not generally possible to orient the binding of antibodies or to attach them covalently. This is one reason why latex particles offer advantages. This will be further discussed in the following section on signal reagents.

TABLE 2   Key Issues in the Choice of Monoclonal *vs* Polyclonal Antibodies for Lateral Flow Applications

Polyclonals	Monoclonals
Fastest to produce in high quantity	Well-defined reagent
Relatively inexpensive to produce	Easy to prepare pure antibody
Can provide very high affinities	Can achieve higher antibody loading on particles and capture lines
Can vary between animals and over time	Take longest to prepare. Typically more expensive
May require extensive purification. Low percentage of total antibody is target specific	May not provide highest affinities.

TABLE 3   IgG-Binding Capacity per Colloidal Gold Particle

Gold Diameter (nm)	IgG Capacity per Particle
1	1–3
5	3
10	12
20	48
30	86–100
40	150–160

Antibody screening methods for lateral flow

It is common practice to use ELISA for screening and selection of antibodies. Typically, this involves coating plates with the molecule of interest.

Although this approach is useful for finding potential antibody pairs and relative ranking of affinities, it is often not predictive of performance in LFIAs. The assay conditions are very different. Typically, these screening assays involve long incubation times. The reactions are therefore often at equilibrium, and antibody surface concentrations in ELISAs are much lower than in LFIAs.

Although initial screening of antibodies may be done with an ELISA or similar format, the antibodies should be tested as early as possible in a format predictive of actual performance in the LFIA.

This is conveniently done using a spotting method, which tests the antibodies and reagents in the assay format in which they will actually need to perform. It is a very simple method and is amenable to screening large numbers of antibodies and assay conditions. It uses a "1/2 dipstick" which is a lateral flow strip without a sample or conjugate pad. The capture antibody is spotted under various conditions to explore immobilization conditions. The antigen is premixed with conjugate either in small tubes or microtiter plates. The strip is then dipped into this solution. This is, of course, a type of mix-and-run format but is predictive of standard LFIA performance with dried conjugates. It is, however, important to perform this testing using the antibodies both as conjugate antibodies and capture antibodies, as premixing the conjugate with the sample affords a much longer conjugate/analyte reaction time than would occur using a dried conjugate, so performance of a pair orientation in the actual system may be different (Fig. 9).
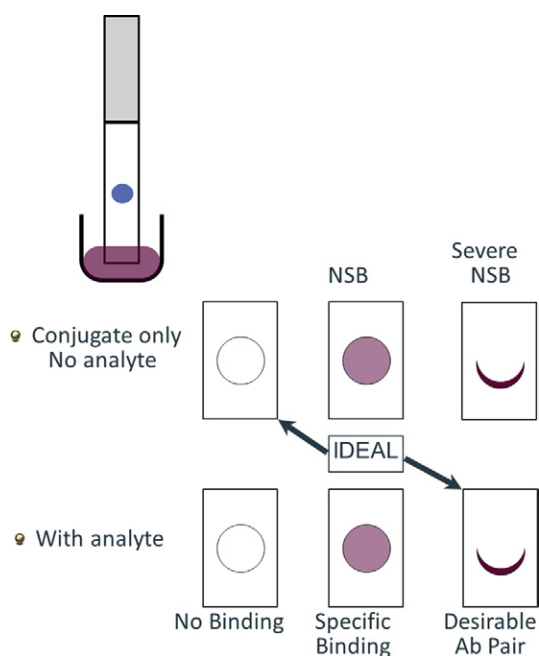

FIGURE 9 Half strip spotting method. *(From Fiechtner, 2009)*. (The color version of this figure may be viewed at www.immunoassayhandbook.com).

Alternative capture reagents for lateral flow applications – aptamer technology

One of the critical elements to be addressed in the design and development of rapid assays for POC is the issue of thermal stability. The majority of LFIAs require controlled storage conditions for optimal stability. Certain assays are completely intolerant of higher temperatures. This is largely a function of the stability of the binding reagents used in the system. This requirement for temperature control, even if the assays do not necessarily have to be kept at low temperatures, imposes cost on the supply chain and also may prevent the use of the test in high-temperature field environments. This is a major issue for diagnostics in developing world applications or in, for example, biowarfare, agricultural, or veterinary testing applications in the developed world.

The use of aptamers represents an alternative to antibodies for assays that require high thermal stability.

Aptamers are oligonucleic acid or peptide molecules that bind to a specific target molecule. Aptamers are usually created by selecting them from a large random sequence pool. More specifically, aptamers can be classified as

- DNA or RNA aptamers. They consist of (usually short) strands of oligonucleotides.
- Peptide aptamers. They consist of a short variable peptide domain, attached at both ends to a protein scaffold.

Nucleic acid aptamers are nucleic acid species that have been engineered through repeated rounds of *in vitro* selection. Aptamers are useful in biotechnological and therapeutic applications as they offer molecular recognition properties that rival that of antibodies. In addition to their discriminate recognition, aptamers offer advantages over antibodies as they can be engineered completely in a test tube, are readily produced by chemical synthesis, possess desirable storage properties, and elicit little or no immunogenicity in therapeutic applications.

In 1990, two labs independently developed the technique of selection: the Gold lab, using the term systematic evolution of ligands by exponential enrichment (SELEX) for their process of selecting RNA ligands against T4 DNA polymerase and the Szostak lab, coining the term *in vitro* selection, selecting RNA ligands against various organic dyes. The Szostak lab also coined the term aptamer (from the Latin, *aptus*, meaning "to fit") for these nucleic acid-based ligands. Two years later, the Szostak lab and Gilead Sciences, independent of one another, used *in vitro* selection schemes to evolve single stranded DNA ligands for organic dyes and human coagulant, thrombin, respectively. There do not appear to be any systematic differences between RNA and DNA aptamers, save the greater intrinsic chemical stability of DNA.

Since the discovery of aptamers, many researchers have used aptamer selection as a means for application and discovery. In 2001, the process of *in vitro* selection was automated by the Ellington lab at the University of Texas at Austin and at SomaLogic, Inc. (Boulder, CO), reducing the duration of a selection experiment from 6 weeks to 3 days.

The SELEX process has been most commonly applied in commercial applications. However, alternative selection methods are coming to market now that increase the efficiency of selection. Companies such as Base Pair Biotechnologies (Houston, TX, USA) and BioAptus (Belo Horizonte, Brazil) have developed methods for rapidly screening aptamer libraries and generating highly purified, high-affinity aptamers in fast turnaround times, greatly decreasing the cost of the reagent.

The primary advantages of aptamers for use in rapid diagnostic applications include

- high thermal stability,
- tailored specificity and affinity,
- ability to generate aptamers to almost any epitope, even nonimmunogenic epitopes,
- scalability of production—once selected, the molecules are simply sequenced and produced.

The use of aptamers in lateral flow technology is under investigation at the time of writing. Commercialization of assays based on aptamers has been limited to date, however, the technology shows promise based on the advantages listed above.

Signal Generation

The signal generation elements involve both the label and the reader or interpretation method used. For POC immunoassays, the majority of detection is still done visually either by eye or using optical readers, however, there is growing use of fluorescent, chemiluminescent, and magnetic field measurement systems. A variety of signal transducers are used, including fluorophores, either particle bound or direct labeled, optical (colloidal gold or colored latex particles) or paramagnetic particles. There has been an industry-wide move to incorporate reader system into POC immunoassay systems, of which, for reasons primarily of sensitivity, availability, and cost, a large proportion are based on the use of fluorescence. In pushing sensitivity lower, nonvisual labels such as fluorescent particles can be expected to generate 1–2 logs greater sensitivity than a typical colloidal gold or colored visual label.

The most commonly used particulate detector reagents in lateral flow systems are colloidal gold and monodisperse latex. The latex particles are available coupled with a variety of detector reagents, including colored dyes, fluorescent dyes, and magnetic or paramagnetic components. The choice of the particulate label and the detector reagent used in a particular lateral flow system are driven by a variety of factors including the following.

- A need for covalent conjugation, typically for stability, sensitivity, or control of variability. Covalent conjugation can yield benefits in all of these areas. Activated latex particles are typically the only choice available for this type of conjugation, as binding of proteins to colloidal gold is typically achieved *via* passive absorption.
- A requirement for quantification. The development of truly quantitative assays in lateral flow formats requires a great deal of effort and care in development and the choice of the basic materials and technologies to be used. If the application requires a reader technology, then available options for label choice expand

to include colloidal gold, colored, or fluorescent latex, and paramagnetic latex particles.

- High sensitivity. In visually read assays, it is often possible to increase sensitivity using colloidal gold instead of colored latex particles due to the smaller size of the gold particles (typically in the range of 20–40 nm as against 100–300 µm for colored latex particles used in these systems) and the higher packing density that can be achieved on a test line as a result. Gold also has a higher color intensity than a typical colored latex particle, which allows for better discrimination of low positives in an assay. In contrast, however, latexes can be produced in multiple colors and can utilize darker colors such as dark blue dyes, which provide greater contrast against the white background of a lateral flow membrane than a colloidal gold. In reader-based assays, it is often possible to generate higher sensitivity, in the region of several orders of magnitude higher in some cases, using fluorescent or paramagnetic particles, than with visual labels, such as colloidal gold or colored latex.

Other label options are available, although they are not commonly used. Options include up-converting phosphors, enzymes, colloidal carbon, and platinum. Paramagnetic particles are also an option. These particles can generate high-sensitivity results, however, for a variety of business and licensing issues, their use is limited.

Common particles, labels, and suppliers

Table 4 lists some of the most common fluorescent dyes used in LFIAs, with excitation and emission wavelengths. Table 5 lists commonly used fluorescent and colored particles.

Improvements in label performance for next generation lateral flow

Traditional approaches to LFIAs involve the dispersion of conjugated particles onto a fibrous conjugate pad such as glass fiber or polyester. These glass fibers are hydrophobic materials that are treated with hydrophilizing reagents in order to make them hydrophilic enough to accept the conjugate. The conjugate is then dried in place on the conjugate pads using high temperatures or lyophilization. The conjugates are typically dried down and stabilized in high concentrations of sugars or polymers.

This process requires multiple steps, each of which inherently contains sources of variability, which in turn result in variability in the test results. The efficiency of release from conjugate pads is typically not high and is always highly variable for particle conjugates. This is a major contributor to the standard coefficient of variation (CV) in signal strength seen in LFIAs, which is in the range of 15–30% strip-to-strip. In order to overcome these issues, a number of alternative approaches have been developed by Diagnostic Consulting Network (DCN).

1. The use of direct fluorescent labeling. As an alternative to the use of particulate labels, it is possible to label the detector reagent with a fluorescent dye directly. Common wisdom indicated that a loss in sensitivity would occur over the use of fluorescent particles. The reasoning behind this was that a

TABLE 4   Commonly Used Dyes in Lateral Flow Applications

Manufacturer	Description	Excitation Wavelength (Ex, nm)	Emission Wavelength (Em, nm)
Invitrogen/Life Technologies	Alexa Fluor® 350	346	442
	Alexa Fluor 405	400	424
	Alexa Fluor 430	433	539
	Alexa Fluor 488	494	519
	Alexa Fluor 514	518	540
	Alexa Fluor 532	530	555
	Alexa Fluor 546	554	570
	Alexa Fluor 555	555	572
	Alexa Fluor 568	578	603
	Alexa Fluor 594	590	617
	Alexa Fluor 610	612	628
	Alexa Fluor 647	651	672
Thermo Fisher	DyLight® 350	353	432
	DyLight 405	400	420
	DyLight 488	493	518
	DyLight 550	562	576
	DyLight 594	593	618
	DyLight 633	638	658
	DyLight 650	652	672
	DyLight 680	692	712
	DyLight 755	754	776
	DyLight 800	777	794
AZCO Biotech	TF1	341	447
	TF2	399	522
	TF3	554	578
	TF4	588	610
	TF5	656	670
	TF6	686	702
	TF7	756	775
	TF8	787	808

fluorescent particle that is in the range of 300–400 nm diameter can contain 20,000–30,000 molecules of a fluorophore. By comparison, an antibody can typically be labeled with only 5–10 molecules. Practice, however, indicates that the loss in sensitivity is minimal if existing at all and that the CV in the system is much reduced. This may be because bound antibody density on the test line can be so much higher with no particles present, compensating for the number of fluorophores per binding event. Release of directly-labeled proteins from conjugate pads is also much higher, as there is less chance of entrapment in the fibers of the materials when no particle is present (O'Farrell, 2010).

2. Lyophilization. Traditional processes involve the drying of particulate conjugates onto conjugate pads using forced air ovens or drying tunnels. This process is inherently nonhomogeneous, resulting in variability in performance, particularly stability and release, of conjugates in standard lateral flow designs. An alternative is the use of lyophilization to create pellets and to store those pellets off-line from the rest of the assay. This means that the conjugates

are not on the strip but are instead dried in another element of the assay, such as in a tube to which sample can be added before delivery of the mixed sample and conjugate to the device.

The removal of the particle from the system brings with it a variety of advantages, including

- improved efficiency of release from conjugate pads,
- improved stability of dried or lyophilized reagents,
- improved reproducibility of release.

An additional advantage of this approach is that the conjugate and sample are premixed, which allows for better performance in antigen assays and avoids potential issues related to slow release from the conjugate pad and lack of active mixing of sample and conjugate. Very high sensitivity and very low CVs (<10%) are possible using this system. On the downside, one result of this approach is that the manufacturing process differs from traditional approaches, requiring the optimization and operation of a lyophilization process, an inherently batch process, which unless carefully designed can become a manufacturing bottleneck. Lyophilization and stabilization of pellets are specialized processes

TABLE 5 Commonly Used Particles in Lateral Flow Applications

		Particles		
Manufacturer	**Description**	**Ex (nm)**	**Em (nm)**	**Surface Chemistry**
Bangs Labs	Ultra violet	360	390	None and COOH
	Plum purple	360	420	
	Glacial blue	360	450	
	Yellow green	441	486	
	Surf green	470	525	
	Dragon green	480	520	
	Envy green	525	565	
	Suncoast yellow	540	600	
	Flash red	660	690	
Invitrogen	Blue	350	440	None, COOH, aldehyde, amine, sulfate
	Blue	365	415	
	Yellow green	505	515	
	Nile red	535	575	
	Orange	540	560	
	Red–orange	565	580	
	Red	580	605	
	Red	580	605	
	Crimson	625	645	
	Dark red	660	680	
	Infrared	715	755	
Merck-Estapor	White	375	420	COOH, amine
	Yellow (X)	468	510	
	Pink (XC)	480	525	
	Red (Z)	515	550	
	Pink (Y)	515	570	
Thermo Scientific	Fluoro-Max Eu	333	613	COOH
Magsphere	Red			COOH, amine
	Yellow–green			
	Blue			
	Orange			
Spherotech	Yellow	460	485	COOH
	Nile red	510	560	
	Pink	560	590	
	Purple	600	615	
	Nile blue	605	650	
	Sky blue	680	705	
Phosphorex	Blue/violet	360	440	COOH
	Green	460	500	
	Orange	540	560	
	Red	660	685	
PolySciences	Bright blue	360	407	COOH
	Yellow green	441	485	
	Yellow orange	529	546	

that are relatively difficult to scale into production volumes for organizations that are unfamiliar with the process. There are, however, service providers available that can optimize these types of processes and provide the lyophilization service on a production scale. Finally, lyophilized pellets, unless carefully designed and stabilized, are highly susceptible to humidity. Many lyophilization processes require storage of the ultimate product in humidity levels as low as 3%. This results in a need for special handling of pellets in a lateral flow production environment and in the inability to store

FIGURE 10 Examples of conjugate and buffer storage devices that can be integrated into cassettes and sample collection devices. *Images provided by Symbient Product Development (CA, USA).* (The color version of this figure may be viewed at www.immunoassayhandbook.com).

the pellets in a cassette in the normal packaging environment, which tends to hold humidity at 20% or below. The key to success is to have a strategy involving careful design of the stabilizers and excipients used in the pellet, such that the pellet can be handled and stored in stable condition at or around 20% relative humidity.

The other disadvantage is that there now may be two or more operator steps required. This can be overcome, however, through some creative design and development strategies. For example, DCN and Symbient Product Development have designed integrated lateral flow devices that incorporate a variety of additional functionality, including onboard reagent storage and integrated sample handling features. It is possible to design cartridges with reagents stored on board, either in lyophilized form or in liquid form. It is therefore possible to design devices that are simple to operate for the user, can still achieve CLIA-waived in the US (CLIA refers to Clinical Laboratory Improvement Amendments of 1988, *see* POINT-OF-CARE TESTING), but require what would be defined as multiple user steps, including sample delivery, conjugate rehydration, and chase buffer delivery (Fig. 10).

Signal Transduction and Analytical Processing

Traditional POC immunoassays were designed to be qualitative, threshold assays, interpreted by the user without the aid of a reader. This method of interpretation brings issues of subjectivity into play and makes it virtually impossible to develop quantitative systems. In clinical and field environments, there is also the possibility of data loss, mistranscription of patient information, and user error. Developing assays with integrated reader systems is a critical feature of many next generation POC assay systems and represents a major technical challenge, particularly for field-based diagnostic platforms.

Commercially available reader systems were developed and are available for use in a variety of environments and applications. Readers may be handheld, portable benchtop (mobile) or true benchtop systems. Multiple optics formats

are also available. The following discussion will be limited to colorimetric (e.g., colloidal gold or colored latex) or fluorescent systems.

In developing instrumentation for application in the field, the goal is to design tests and devices that have the appropriate level of complexity as required by the biology of the analysis process. Automated reading of these tests makes reading the test result relatively easy and also reduces the chance for human error.

Numerous reader technologies exist for use in lateral flow applications, produced by companies such as Qiagen Lake Constance (Germany), Axxin Inc. (Australia), Hamamatsu (Japan), Alverix (USA), and LRE Medical (Germany). These units are ideal for application in many first world environments, being robust, relatively low-cost, calibrated, high-performance units with associated customer service and support available. However, for developing world applications, these readers are generally too complex and expensive, and the supporting infrastructure for calibration, maintenance, and operation is not in place.

An alternative approach, still in the early stages of development, is the use of smart phone camera capabilities to transform analog test results into digital format. A further technology advancement is the development of analysis applications and algorithms that then interrogate the digital data to provide a usable test result that can also include treatment options. An example of such a smart phone-based approach is the AQ Droid from Clearbridge BioLoc (Singapore) and the SkanSmart and SkanEasy products from Skannex (Norway). These products provide reader solutions that can be applied in the field and are leveraged off existing optics and communications technologies. These generic approaches to the reading of tests in the field face some challenges, however, including platform obsolescence issues, a lack of coordination with test developers, and calibration issues. Regulatory issues will also inhibit the adoption of this type of technology in many developed world markets. As a result of these issues, the application of readers in medical diagnostic applications will likely remain limited to dedicated reader systems, designed specifically for the application, for some time to come.

Available optics options for lateral flow applications

Imaging systems

The primary advantage of imaging systems is that they produce a full picture record of the read area containing the test and control lines. This can be of importance in a number of ways, including archiving of results for medical or law enforcement applications, and averaging of line intensities over the full width of the strip, which can be used to advantage if there is inhomogeneity in the line development—a feature particularly useful in qualitative applications. For robust readers, intended to be field deployed, the absence of moving parts in these systems can be a particular advantage. Additionally, many imaging systems can be customized and optimized to the label used by a specific customer in order to reduce noise and increase sensitivity of detection. Such customization allows instruments to be offered that read fluorescent labels, including europium-based particles or fluorescent molecules.

Image generation and image processing technology have improved significantly in parallel with advances in digital camera technology. Several companies, including Detekt Biomedical, LLC (Austin, TX), Alverix (San Jose, CA), Axxin Inc. (Victoria, Australia), and Skannex (Hoenefoss, Norway), have incorporated low-cost, high-performance imaging hardware into their dedicated devices. Other companies use existing smart phone cameras coupled with image analysis software to generate high-fidelity results in lateral flow systems. All couple the imaging capability with high-performance image analysis software that can identify the presence and position of test and control lines, determine intensity, subtract background, and compare results to preprogrammed calibration curves and/or cutoff criteria for positive/negative determination.

The disadvantages in these systems come from a number of sources. Older CCD-based technologies are higher cost and difficult to miniaturize. Newer CMOS-based technologies, although cheaper, can be lower performing in terms of image fidelity. Although true for all of the available technologies, the cost/volume ratio for imaging systems tends to be critical. Considered to be among the major disadvantages of systems that utilize generic platforms, such as smart phones, is the issue of platform obsolescence. Software, firmware, and hardware for such platforms are constantly evolving, resulting in regular obsolescence, leaving critical elements of the supply chain outside the reach of the service provider. Certain reader providers have overcome this issue by stockpiling the base units around which their technology is based, guaranteeing supply to their customers in this way. Other providers include obsolescence in the design phase, allowing them to carefully select components with a practical life cycle, and to plan for future component substitution (Figs 11 and 12).

Scanning systems

A variety of suppliers including Qiagen Lake Constance (Stockach, Germany), LRE (Munich, Germany), and Hamamatsu (Tokyo, Japan) provide lateral flow readers based on scanning optics. Basic options are point scanning systems, where the area of the scan is small, making up only 10–20% of the developed line width, typically, compared to line scanning, where the scan is completed across the entire width of the line or very close thereto. With the point scan, it becomes critical to have strips that develop very evenly across their entire width, which pushes much of the responsibility onto the strip developer and manufacturer to ensure that aberrant results are not generated due to improper flow characteristics in the strip. This is an issue that can be generally avoided with proper design and control of the strip manufacturing process. The advantage of the scanning systems comes in their low cost and low complexity (Figs 13 and 14).

Summary

In general terms, solutions exist for the design, development, and implementation of reader systems in lateral flow

FIGURE 12 Benchtop imaging system for lateral flow tests from Axxin Inc. (The color version of this figure may be viewed at www.immunoassayhandbook.com).

FIGURE 13 ESEQuant™ lateral flow strip reader from Qiagen Lake Constance. (The color version of this figure may be viewed at www.immunoassayhandbook.com).

FIGURE 14 cPOC™ lateral flow strip reader from LRE. (The color version of this figure may be viewed at www.immunoassayhandbook.com).

FIGURE 11 Handheld image capture lateral flow readers from Detekt Biomedical, LLC. (The color version of this figure may be viewed at www.immunoassayhandbook.com).

applications. The key to the implementation of such systems is in the careful setting of specifications and choice of partner. It should be remembered that the reader is, however, only one portion of an optimally performing assay platform, along with the test strip and the device it is being run within, and all three elements must be optimized together for best effect.

Device Design Considerations for Next Generation Performance

In designing a complete LFIA system that has high-level performance and importantly, freedom to operate in a commercial environment, it is critical to consider the overall device design. Some creativity in design can lead to enormous performance benefits. Typical areas for improvement over standard strip performance include the ability to multiplex and the ability to generate results with low CV, allowing for quantification. Each will be considered below with some examples of possible design approaches.

Quantification and reader integration

There are numerous elements in cassette design that come into play when considering quantification in LFIAs. These include the following:

Control of sample addition

In many systems, it becomes critical that an accurate sample volume be quantitatively delivered to the strip. This sample may have been pretreated, for example, by filtration or dilution. Careful consideration of the tolerances of delivered volume and concentration should be made, tolerances for the assay established during feasibility, and the tolerance of the assay should determine many of the design decisions made in the sample handling steps.

Control of flow

There are numerous engineering considerations in ensuring proper and consistent flow in cartridges. The first rule to be applied is that unless all materials are the same between different strips, the use of the same cartridge across multiple product lines requires careful evaluation. Off-the-shelf parts should never be used. Materials and processes commonly used for lateral flow production have relatively large tolerances that must be accounted for in the design of the cartridge. Key considerations include

- Fit of test strip into cartridge – tolerance of materials and laminations.
- Does sample pad allow for adequate compression to prevent leakage?
- What are the tolerances of overlaps/laminations?
- What are the tolerances of the line positions?

It is important to begin by generating engineering drawings of the actual strip, a step that is commonly overlooked by strip developers. This gives the engineers a clear idea of the variability in the system and the key tolerances, they have to account for in the plastic device (Fig. 15).
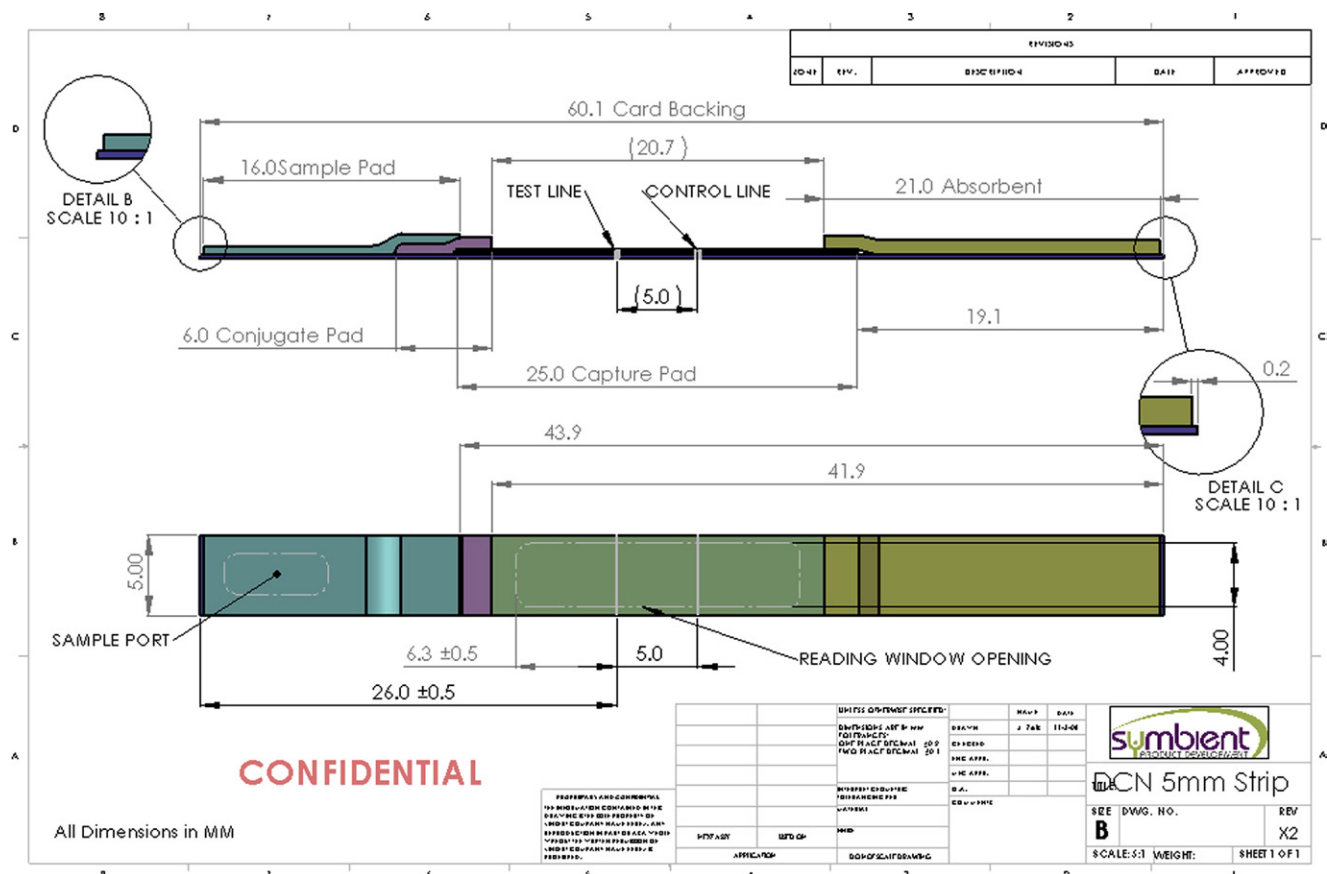

FIGURE 15 Example of an engineering drawing for a lateral flow strip accounting for key specification and tolerances. *Source: Diagnostic Consulting Network/Symbient Product Development.* (The color version of this figure may be viewed at www.immunoassayhandbook.com).
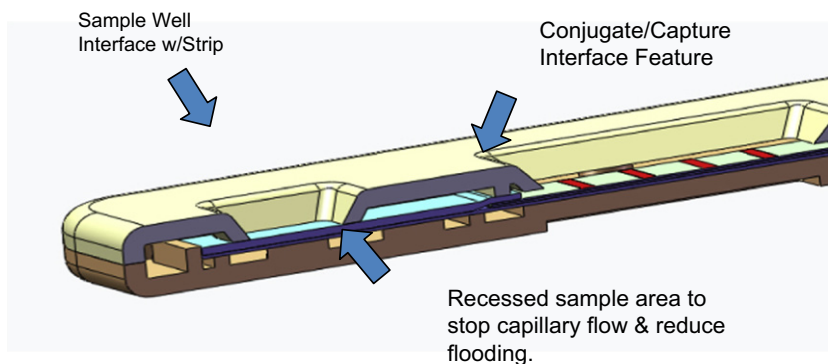
FIGURE 16 Key engineering features in a lateral flow cassette. *Source: Diagnostic Consulting Network/Symbient Product Development.* (The color version of this figure may be viewed at www.immunoassayhandbook.com).

The cassette contains a set of engineered features that are key to ensuring the controlled and consistent flow of fluid in the system. Some of these are shown in Fig. 16.

Ensuring that the entire sample added to the sample well is absorbed into the sample pad without flooding is the first key step. Particularly when the device is to be interrogated by a reader, even line development across the width of the strip is critical. The pressure points at the overlaps between pads are critical to ensuring that evenness of flow.

Reader Integration

For reader integration, there are another set of key considerations, including the tolerances of line positions, which are controlled by the lamination process and by the positioning of the strip in the device and the device in the reader. The manufacturing process for the strip must be controlled to prevent serious drift in the line position during striping and lamination. The cassette must hold the strip in the correct position, and features in the drawer on the reader need to hold the device in the correct orientation relative to the read head. Also critical for most optical systems is the shape of the read window and how that affects illumination of the strip. The cassette should not cast shadows when illuminated.

Multiplexing

If there is an intention to multiplex, early consideration should be given to whether to attempt to multiplex on the strip (i.e., multiple analytes on one strip) or whether to generate multiple strips within a single cartridge. Whether a single strip can be used depends completely on the assays and reagents, and whether the analytes can be presented to each assay using a single set of conditions that allows each set of

reagents to function appropriately. This decision can impact the choice of reader, the cassette designs and tooling, and the overall approach to product line development (Fig. 17).

CONCLUSIONS: FUTURE DIRECTIONS FOR LATERAL FLOW

Extrapolating the future requirements and direction of the rapid testing markets based on past performance and trends is difficult and largely an exercise in futility. For example, there are tectonic shifts underway in the clinical rapid assay/POC marketplace, including the way in which products are bought and paid for, in regulation, the emergence of new and powerful companies from within and outside the traditional diagnostic marketplace, and the appearance of completely new markets, such as personalized medicine and companion diagnostics. For all of these reasons, it is difficult to predict where the evolution of the market will take us in the coming 5–10 years.

However, from a technology perspective, there is a relatively clear form to the way lateral flow is evolving. It is likely that the focus on the implementation of continuous improvements with the aims of better sensitivity, reproducibility, quantification, and multiplexing capability, will continue. The evolution of applications into areas such as consumer diagnostics will continue to drive the move toward integrated design features that lead to intuitive use by minimally or untrained users. Additional applications will also continue to be found. One prime example spanning a variety of potential market segments is **nucleic acid lateral flow (NALF)**.

The goal for next generation assays is to achieve the sensitivity of a molecular technique coupled with the robustness, ease of use, simplicity, and cost of a lateral flow test strip. Standard approaches to the laboratory detection of specific strands of DNA or RNA for diagnostic applications involve the application of inherently labor-intensive, complex workflow elements, many of which require automation and high levels of quality control, particularly in quantitative applications, to produce validated results. Reducing this complex workflow to a simple disposable cartridge or simple set of easily-performed steps represents a huge challenge to the implementation of molecular
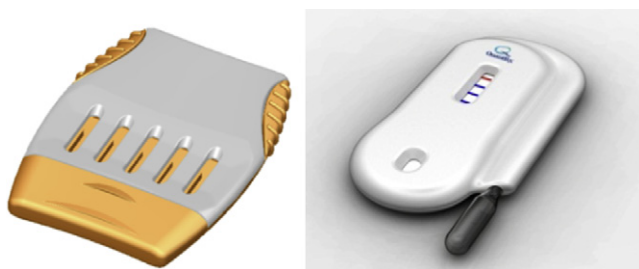

FIGURE 17 Multiplex cassettes: single strip *vs* multiple strips in one cassette. *Source: Symbient Product Development.* (The color version of this figure may be viewed at www.immunoassayhandbook.com).

tests at the POC. Doing so means addressing the fundamental way in which each of the steps in the workflow of a molecular diagnostic test is performed and integrated into a test device.

One key element of this simplification approach is the use of noninstrumented detection approaches. NALF is a method that has been successfully used in a number of systems. Methods for preparing and manufacturing NALF devices are relatively straightforward and well proven (for example, see Piepenburg *et al.*, 2006). Implementation on an industrial scale has proven more difficult, however, a limited number of systems have come close to commercialization, including the BioHelix ExpressStrip (BESt) and SAMBA (Simple amplification based assay) SAMBA from Diagnostics for the Real World (Cambridge, UK) (Rosen and O'Farrell, 2011). Progress is continuing at a rapid rate, and it is expected that NALF will become a standard tool in next generation POC nucleic acid testing.

This is just one example of an area in which lateral flow technology is continuing to evolve, however, it is a prime demonstration of the flexibility of this technology.

The World Health Organization Sexually Transmitted Diseases Diagnostics Initiative (SDI) developed the ASSURED criteria as a benchmark to determine if tests address disease control needs. These criteria have since become widely applied as a useful benchmark for practical diagnostic technologies intended for use in most decentralized or rapid-testing environments. These key criteria are Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end users.

Lateral flow technology remains the only widely available assay format that can address most, if not all, of the ASSURED criteria while also demonstrating flexibility of application and a capability for performance fidelity that approaches that of much more complex diagnostic technologies. As a result of this unique confluence of characteristics, it is likely that this assay format will have a place in the rapid testing arsenal for many years to come.

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