

Chapter 1

Evolution in Lateral Flow–Based Immunoassay Systems

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1.1 Introduction: History of Membrane-Based Point of Care Immunoassays

The development of the rapid, immunochromatographic test strip, also known as lateral flow immunoassay (LFIA), is the result of convergence of several threads that can be traced back to the 1950s. However, the concept of rapid diagnostic tests based on body fluids dated back significantly further. Documented evidence of saliva- and urine-based diagnostics existed several thousand years ago. The ancient Chinese were among the first documented users of saliva-based diagnostics. One widely used practice involved the use of saliva as a rapid determinant of guilt. In the “Rice Test”, the inability to generate enough saliva to swallow a handful of rice was considered sufficient evidence for conviction. In this way, a rapid result was generated, but often with a poor prognosis for the subject. One of the earliest written records of a urine-based diagnostic test for pregnancy can be found in ancient Egyptian documents. There, a test was described whereby a potentially pregnant woman could urinate on wheat and barley seeds over the course of several days. The results: “If Barley grows, it means a male child. If wheat grows, it means a female child. If both do not grow, she will not bear at all” [1]. The interest in urine as a rapid diagnostic medium for a variety of ailments continued through the Middle Ages, with the advent of the so-called piss-prophets in Europe, who claimed to be able to differentiate many different conditions from the color of urine. Along with many medical concepts of the time, success typically varied. Despite best efforts through the ages, it was not until the mid-twentieth century that the majority of rapid diagnostic methods gained real predictive value.

The technical basis of the lateral flow immunoassay was derived from the latex agglutination assay, the first of which was developed in 1956 by Plotz and Singer [2]. In the same period, plate-based immunoassays were being developed.

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The first radio-immunoassay (RIA) was invented by Berson and Yalow in the 1950s [3]. The enzyme immunoassay (EIA) was introduced in the 1960s, bringing with it significant advantages, including the replacement of radioisotopes with enzymes, faster reaction times, higher specificities, and longer shelf-life than RIA. The basic principles of the lateral flow technology continued to be refined through the early 1980s and were firmly established during the latter years of that decade, with the filing of several major patents on this technology format by companies such as Becton Dickinson & Co. and Unilever and Carter Wallace [4–6]. Since then, at least another 500 patents have been filed on various aspects of the technology.

The main application driving the early development of the solid phase, rapid-test technology was the human pregnancy test, which represented continual historical interest in urine testing for medical diagnostic purposes. This particular testing application made great strides in the 1970s, as a result of improvements in antibody generation technologies and significant gains in understanding of the biology and detection of human chorionic gonadotropin (hCG), derived largely from the work performed by Vaitukaitis and co-workers [7]. However, to fully develop the lateral flow test platform, a variety of other enabling technologies were also required. These include technologies as diverse as nitrocellulose membrane manufacturing, antibody generation, fluid dispensing and processing equipment, as well as the evolution of a bank of knowledge in development and manufacturing methodologies. All of these elements were required to render a mélange of complex chemicals, biologicals, papers, polymers, people, and processes into a simple and easy-to-use test, which is able to adequately perform to provide prognostic results in a variety of critical applications. Many of these facilitative technologies had evolved throughout the early 1990s, to the point where many are now mature, off-the-shelf technologies. As a result of the early work in all of these areas, the first lateral flow products were introduced to the market in the late 1980s. Since then, the technology, its applications, and the industry have all continued to evolve. As of 2006, over 200 companies worldwide are producing a range of testing formats, with a total value of approximately \$2.1 billion dollars (USD) in major market segments (Stratcom, personal communication) (also see Chapter 2). The application of the technology has expanded well beyond clinical diagnostics to areas as diverse as veterinary, agriculture, biowarfare, food, environmental health and safety, industrial testing, as well as newer areas such as molecular diagnostics and theranostics.

The purpose of this chapter is to introduce readers to many of the key elements of the lateral flow immunoassay, to describe the basic process of producing a lateral flow immunoassay, and to understand why it has achieved such broad penetration in so many market areas. This chapter will also discuss the limitations of the current technology and how this technology must evolve to meet ever more demanding market requirements.

1.2 Architecture of a Lateral Flow Immunoassay

Figure 1.1 shows the typical configuration of a lateral flow immunoassay. Traditionally designed assays are composed of a variety of materials, each serving one or more purposes. The parts overlap onto one another and are mounted on a backing card using a pressure-sensitive adhesive. The assay consists of several zones, typically constituted by segments made of different materials. These will be briefly explained here. When a test is run, sample is added to the proximal end of the strip, the sample 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, where a particulate conjugate has been immobilized. The particle can typically be colloidal gold, or a colored, fluorescent, or paramagnetic monodisperse latex particle (see Chapter 5). This particle has been conjugated to one of the specific biological components of the assay, either antigen or antibody depending on the assay format (see Chapter 4). The sample re-mobilizes the dried conjugate, and the 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 component of the assay has 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 the 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. The assay formats can be either direct (sandwich, Fig. 1.2a) or competitive (inhibition, Fig. 1.2b) and should be able to accommodate qualitative, semi-quantitative, and, in limited cases, fully quantitative determinations. Direct assays are typically used when testing for larger analytes with multiple antigenic sites, such as hCG, Dengue antigen, or

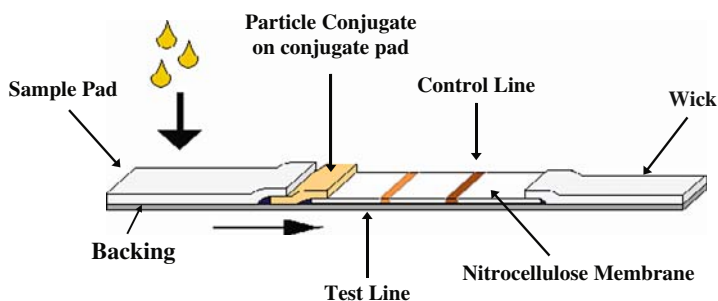


Fig. 1.1 Typical configuration of a lateral flow immunoassay test strip

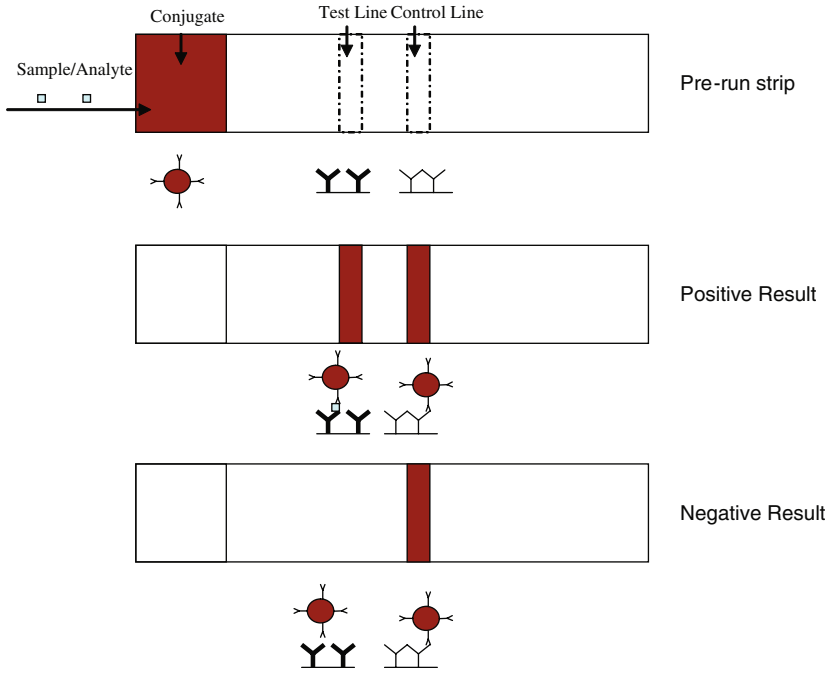


Fig. 1.2a Direct solid-phase immunoassay

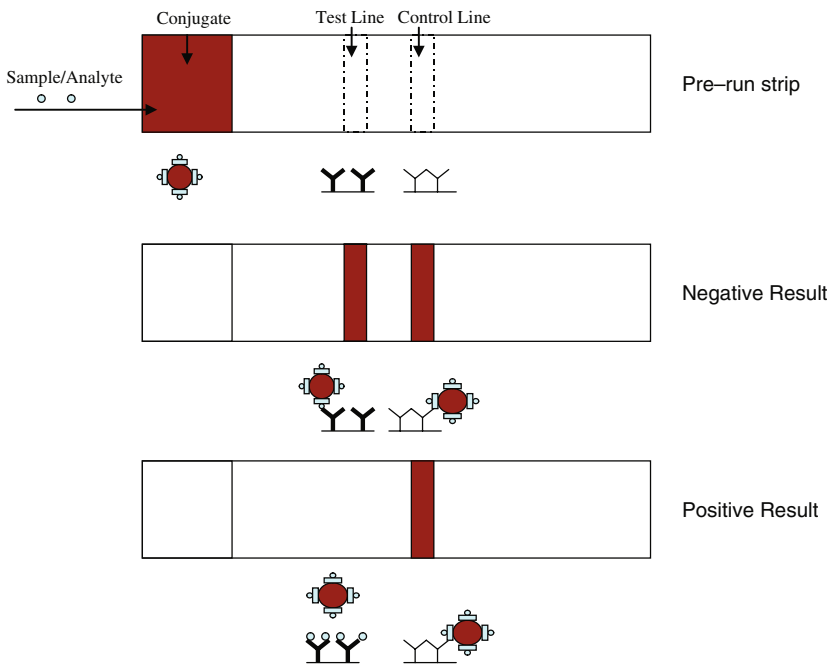


Fig. 1.2b Competitive solid-phase immunoassay

human immunodeficiency virus (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 antibody in the particulate 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.

1.3 Utility of the Lateral Flow Immunoassay Technology: Advantages and Issues

Lateral flow immunoassays represent a well-established and very appropriate technology when applied to a wide variety of point-of-care (POC) or field use applications. The advantages of the lateral flow immunoassay system (LFIA) are well known:

- Established mature technology
- Relative ease of manufacture – equipment and processes already developed and available
- Easily scalable to high-volume production
- Stable – shelf-lives of 12–24 months often without refrigeration
- Ease of use: minimal operator-dependent steps and interpretation
- Can handle small volumes of multiple sample types
- Can be integrated with onboard electronics, reader systems, and information systems
- Can have high sensitivity, specificity, good stability
- Relatively low cost and short timeline for development and approval
- Market presence and acceptance – minimal education required for users and regulators

Critical among these advantages are the POC nature and a very broad range of applications that can be brought to market extremely quickly and for a relatively small investment. These are advantages that few other putative POC technologies currently in development, including sensor- and array-based technologies, can claim to share. While innovation in microfluidics, biosensor, and multiplexed arrays continues at an increasing rate, those technologies typically require long development cycles, careful market selection, market education, and large investment in technology and infrastructure development in order to make significant impacts in most diagnostic marketplaces.

Traditionally designed lateral flow immunoassays, however, have also suffered from performance limitations, most notably sensitivity and reproducibility. Some of these issues are listed below:

- Unclear patent situation (see Chapter 11)
- Miniaturization of sample volume requirements below microliter level
- Multiplexing: simultaneous analysis of multiple markers difficult
- Integration with onboard electronics and built-in QC functions challenging
- Sensitivity issues in some systems
- Test-to-test reproducibility challenging – limits applications in quantitative systems

These limitations have been exacerbated by the continuing use of traditional manufacturing practices and materials, labels, and visual detection systems. In recent years, market pressures have led to the development of a range of new materials, reagents, detection methods, reader systems, and manufacturing process technologies that together yield the potential to significantly improve the performance of lateral flow immunoassays [5]. Some of these elements will be discussed later in this chapter.

Despite issues of perceived or real performance limitations, lateral flow immunoassays have achieved broad penetration in a variety of markets. Figure 1.3 lists the market segments in which lateral flow immunoassays are already in production or are known to be in development. The manufacturing of lateral flow assays that meet the requirements of some of these market segments, however, may represent new challenges. As applications expand,

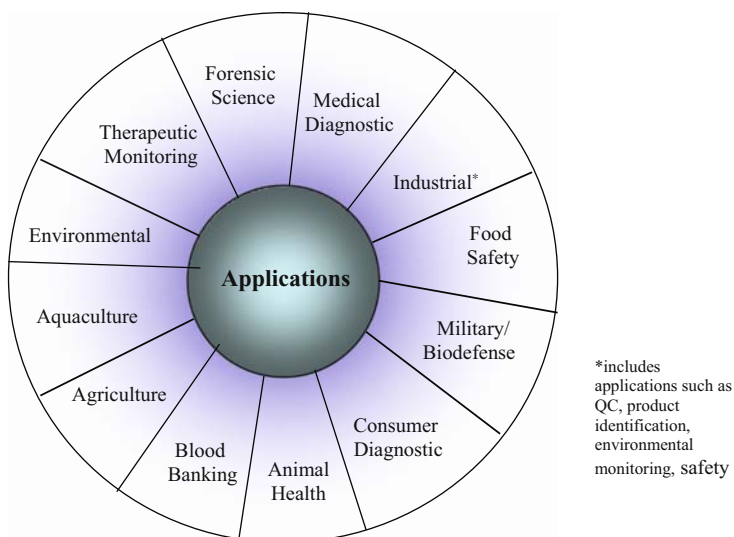


Fig. 1.3 Market segments for LFIA and other point-of-care or field-use technologies

demands on the technology increase, requiring improvements in sensitivity, reproducibility, and manufacturability. Quantification and objective read/record technology, often linked to laboratory information systems (LIS), are being developed. Below is a list of features that are considered desirable for next generation PCOC technologies:

- Rapid and easy to use
- Use small volume of sample, appropriately transferred to the assay without contamination
- Cost-effective to manufacture and use
- Can be manufactured in high volume
- Produce clearly presented and easily interpreted results. Produce high-sensitivity results with low constant of variance (CV)
- Enable quantification
- Integrate to objective read/record technology with built-in connectivity
- Enable multiplexing
- Assays and technology must demonstrate benefit and fulfill a need

In order to meet these demands, there is a growing need for improved materials, assay technology, reader technology, and manufacturing processes. There is also a growing requirement for a more multidisciplinary approach to lateral flow assay development. In the sections below, the elements of the lateral flow technology that present the most challenge to the development of highly reproducible assays will be discussed in the context of the individual components of the assays and the manufacturing methodologies employed in their production.

1.4 Commonly Used Materials and Processes in Lateral Flow Immunoassay Development and Manufacturing

The common components of a lateral flow assay and the standard materials used for each component are considered below. Processing methodologies for each component will also be discussed in relation to how the material drives the manufacturing and development process for typical lateral flow assays.

Figure 1.4 outlines a generalized manufacturing process for traditional lateral flow test strips. The materials and processes typically used for the 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 LFIA.

1.4.1 Assay Components

1.4.1.1 The Membrane/Analytical Region

Purpose: The purpose of the analytical region in a lateral flow immunoassay is to bind proteins at the test and control areas and to maintain their stability and

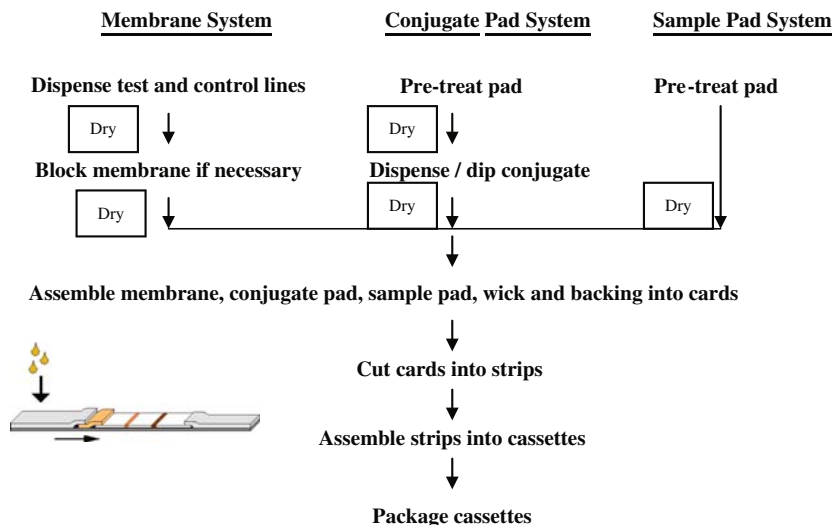


Fig. 1.4 Outline of a typical lateral flow immunoassay manufacturing process [8]

activity over the shelf-life of the product. When the strip is run, it must accept the conjugate and sample from the conjugate pad, flow them consistently to the reaction area, allow the reaction at the test and control lines to happen, and allow excess fluids, label, and reactants to exit without binding.

Material: The material of choice in the vast majority of lateral flow immunoassay systems has historically been nitrocellulose (see Chapter 6). Several attempts have been made to introduce other material types into the market, including nylon and polyvinylidene fluoride (PVDF) membranes. However, those attempts have had limited success, apparently due to factors including cost, limited utility, the need for education regarding new chemistry and processing requirements, and resistance to change due to the large bank of existing experience in the use of nitrocellulose [8].

Nitrocellulose, while extremely functional, is not an ideal matrix for an analytical membrane in LFIA. It does have certain characteristics that make it useful, and it remains the only material that has been successfully and widely applied in this way to date. These characteristics include relatively low cost, true capillary flow characteristics, high protein-binding capacity, relative ease of handling (with direct cast, or backed membranes), and a variety of available products with varying wicking rates and surfactant contents. However, the material also possesses a variety of characteristics that make it imperfect for this application. These include imperfect reproducibility of performance within and between lots, shelf-life issues, flammability (primarily in unbacked membranes), variable characteristics

due to environmental conditions, such as relative humidity and being subject to breakage (if unbacked), compression, and scoring during processing. As a result of these issues with the material, developers and manufacturers spend a considerable amount of time and effort in optimizing chemistries that overcome some of the inherent material issues and in developing manufacturing processes that guarantee adequate performance over the entire shelf-life of the product. Careful control of the key processes of dispensing, dipping, and drying, and attention to chemical and biological treatment of the membrane in order to prevent the introduction of additional variation into the finished product are critical to success.

Flow Characteristics: In order to function as the reaction matrix in a lateral flow immunoassay system, the materials must be hydrophilic and have consistent flow characteristics. Nitrocellulose as a base material is hydrophobic, and is made hydrophilic by the addition of rewetting agents during the membrane production process. These rewetting agents are surfactants, and the type, amount used, and addition methods of surfactant differ from manufacturer to manufacturer and also from brand to brand within a manufacturer. These factors can affect the performance of the assay initially and over time. Not every protein is compatible with every surfactant. This is one reason for screening multiple membrane types during development. The flow characteristics of nitrocellulose membrane change over time, primarily due to desiccation of the membranes upon storage. Nitrocellulose membranes can be envisaged as a sponge, with the pores of the sponge being held open by water. If that water is removed, the pores collapse, disrupting the ability of the membrane to wick fluids through it. This results in changes and inconsistencies in flow rates over time. As speed directly affects assay sensitivity, extended run times can produce false positive results. This is a major contribution to the variability in lateral flow immunoassays.

Critical to the proper performance of a lateral flow immunoassay system is the requirement that it binds reactants only at the desired locations, namely the test and control lines. The protein-binding capacity of a membrane, its interactions with proteins, and the kinetics of the protein-binding process are the parameters that determine the appropriateness of a given set of proteins for the membrane and the sensitivity of the resulting diagnostic tests. Proteins bind to nitrocellulose through a combination of electrostatic, hydrogen, and hydrophobic forces. One of the key elements to the production of sensitive and reproducible assays is the consistent immobilization of immunologically active proteins to test and control lines. It is known that a majority of the proteins lose much of their immunological activity after binding passively to the membrane surface, due to their inability to bind covalently or directionally to nitrocellulose. The commonly accepted model for binding of protein to nitrocellulose is that proteins are initially attracted to the membrane surface by electrostatic attraction. Long-term bonding is

then accomplished by a combination of hydrophobic and hydrogen bonds. Many factors affect the binding process, and these must be considered when developing assays and processing nitrocellulose membranes. Some of these factors are listed below:

(i) Reagent choices [9]

- Non-specific proteins: bulking proteins [e.g., bovine serum albumin (BSA), casein] compete for binding sites
- Materials that interfere with hydrogen bonding: Formamide and urea interfere with hydrogen bonding
- Materials that interfere with hydrophobic interactions: Tween and Triton interfere with hydrophobic bonding
- Polymers such as polyvinyl acetate (PVA), polyvinylpyrrolidone (PVP), and poly-ethylene glycol (PEG) interfere with protein binding by a combination of these effects

(ii) Environment

- Humidity should be optimized for binding (25–50% relative humidity at room temperature)

(iii) Processing Methods

- Dispensing methods: Contact tip versus non-contact will have effects on how protein binds or spreads through the membrane
- Drying methods: Forced air oven at elevated temperature versus ambient drying conditions. Drying time and methods can affect the re-arrangement and activity of proteins on the membrane

Stability: The membrane must not destabilize bound proteins at test and control lines for entire shelf-life or change its flow characteristics in that period.

Membrane Processing: Nitrocellulose must undergo several processes before integration into the final device. These include deposition of test and control line proteins using quantitative dispensers, drying using forced air ovens at elevated temperature, and immersion processes for blocking. To lay down the test and control line proteins, the membrane is striped with proteins using either contact or non-contact dispensing systems, and is blocked thereafter to control and stabilize the flow-rates and hydration characteristics and to prevent non-specific binding. The dispensing method used for the test and control lines must be as quantitative as possible and should not vary with material hydration or absorption characteristics. Non-contact dispensing methods provide the best solution for quantitatively dispensing proteins onto nitrocellulose. The purpose of blocking a nitrocellulose membrane is to prevent the binding of proteins and labeled conjugate to the membrane at areas other than the test and control lines. Blocking also serves other functions, including maintenance of hydration of membranes, modification of wicking rates, and stabilization of test and control line proteins. Blocking is typically performed by immersion of the membranes in a

solution containing proteins, surfactants, and polymers, and is a relatively uncontrolled process. The blocking method must be carefully controlled to produce optimal performance in the final product over its entire shelf-life. Drying is subsequently performed by a combination of blotting to remove surface fluids and with forced air at elevated temperatures to dry. Again, this drying process must be carefully optimized to minimize variation in the final product.

Availability and Choice: The correct combination of membrane types and specific proteins is an important factor for the success of a functional test. Different nitrocellulose membranes can vary considerably in terms of performance characteristics when used with different proteins. Thus, a variety of suppliers and brands of nitrocellulose membranes are available. Performance of the membrane is typically defined by factors such as the polymer type used in the membrane, the pore size, the surfactant type, quantity, and the method of surfactant application. Pore sizes of the membrane used in lateral flow immunoassays range from a nominal 8 to 15 microns, although pore size is a non-exact descriptor in the case of nitrocellulose membranes. The polymeric structure does not actually create pores, but rather a tortuous sponge-like pathway for fluid and particle movement. “Wicking rate” or “capillary rise time” is a more appropriate measure of membrane flow characteristics than pore size. Capillary rise time is defined as the length of time required for a fluid front to traverse a 40 mm width of membrane and is a manufacturer-defined specification for nitrocellulose membranes. The choice of wicking rate is important to the kinetics and speed of development of the assay and will have critical effects on assay performance and sensitivity.

1.4.1.2 The Conjugate Pad

Purpose and General Characteristics: The role of the conjugate pad in a lateral flow immunoassay is to accept the conjugate, hold it stable over its entire shelf-life, and release it efficiently and reproducibly when the assay is run. In practice, variations in conjugate deposition, drying, and release from the membrane constitute major contributions to the coefficient of variation (CV) in assay performance. Assay sensitivity can also be adversely affected by poor conjugate mixing and release from the conjugate pad. Depending on the system, some may favor fast release while others favor slow release of the conjugate. However, the release must always be consistent. Because of the nature of the materials used, it is often necessary to pre-treat conjugate pads to ensure optimal release and stability. Pretreatment is performed by immersion of the pad in aqueous solutions of proteins, surfactants, and polymers, followed by drying. This process, similar to membrane dipping and drying described above, can be performed either in manual batch mode or in continuous inline mode, the latter giving the best opportunity for homogeneous processing of entire batches of materials.

The addition of conjugates to the treated pad is a critical step for the final performance of the test. Two methods are typically used. The first is immersion of the treated conjugate pad into the conjugate suspension. The second is

dispensing with quantitative non-contact dispensers such as the BioDot AirJet Quanti 3000 (see Chapter 8, Fig. 8.4). With respect to the conjugate system, the choice of labels and conjugation methods are important. The most commonly used labels include colloidal gold and monodisperse latex, tagged with either a visual or a fluorescent dye (see Chapter 5). The labels can be covalently or passively coupled and can be read quantitatively. Covalent coupling can be crucial to the ability to perform quantitative assays due to the inherently more stable bonds formed between the ligand and the particle as opposed to passive adsorption methods.

Material: The materials of choice are glass fibers, polyesters, or rayons.

Flow Characteristics: For best results, the materials must be hydrophilic and allow rapid flow rates. Most materials used in lateral flow immunoassay systems are very hydrophobic in nature, and must be treated to make them hydrophilic. This is done during the manufacturing of the assay rather than by the material manufacturer, although there are exceptions to that. Most notably are the Accuflow series of pretreated conjugate pads produced by Whatman (Kent, UK). This treatment involves the immersion of the pads in a solution of proteins, polymers, and surfactants, followed by drying at high temperatures as described earlier.

Release Characteristics: The conjugate pad must release the conjugates efficiently and reproducibly over the shelf-life of the product. Typically, some variation in release may occur due to the nature of binding of the particle conjugate to the fibers of the material. It is important during assay optimization to generate stabilization chemistries that minimize this effect and create the most efficient release of particles possible.

Stability: The conjugate pad must not destabilize the conjugate over the entire shelf-life (up to 2 years). Typically, some destabilization does occur, due to the binders present in the majority of these materials. Assay optimization therefore involves the testing of multiple materials for compatibility with the protein-particle conjugate being used.

Manufacturers and Manufacturing Issues: Most commonly used products are from Whatman (Kent, UK), Ahlstrom (Helsinki, Finland), Pall Gelman (East Hills, NY, USA), and Millipore (Bedford, MA). The conjugate pad system is responsible notably for the majority of variations in lateral flow immunoassays. Variation in the material can lead to inconsistent uptake of the pretreatment liquids and conjugates, destabilization of the conjugates, poor release of the conjugate, and binding of conjugates to the hydrophobic fibers. Great care must be taken during manufacturing in optimizing the conjugates, the pad pretreatment process, and the conjugate deposition process to minimize these effects.

1.4.1.3 The Sample Pad

Purpose: One of the major advantages of the lateral flow concept is that these assays can be run in a single step with many different sample types in a variety of

application areas. Sample types can be as diverse as whole blood from a post-partum mother, a sputum sample from a potential TB sufferer, or a sample of ground beef from a bulk container. Much of the burden of making those samples compatible with the rest of the assay system falls on the sample application pad. The role of the sample pad is to accept the sample, treat it such that it is compatible with the assay, and release the analyte with high efficiency. Sample treatments include the filtering out of particulates or red blood cells, changing the pH of the sample, actively binding sample components that can interfere with the assay, and disrupting matrix components, such as mucins, in order to release the analyte to the assay. The material chosen to fulfill any or all of these functions can have a great effect on assay performance due to the inhomogeneity of many available materials and the type of binders they contain. The method of pad pretreatment is typically via immersion and drying as with the conjugate pad and, if such treatment is required, the method must be carefully designed to avoid introducing sources of variation, including buffer concentration gradients and edge effects upon drying.

Material: The materials used for the sample pad depend on the requirements of the application. Examples of such materials are cellulose, glass fiber, rayon, and other filtration media.

Capacity: The sample pad material must be treated with assay buffer and other components and dried prior to use. It must also be able to accept all of the sample volume applied to it in a controlled way, thereby helping to channel fluids into the assay materials rather than allowing flooding or surface flow.

Strength: The sample pad material should be strong enough to be handled in manufacturing. An important consideration is tensile strength while wet. If this material is to enter high-volume production, it must endure the tension, without breaking, from a reel-to-reel production system while being immersed in a tank of fluid. The immersion of the pad occurs as part of the pad pretreatment, where the pads are impregnated with an assay buffer containing pH buffer, surfactants, blocking reagents (if required), additives, and other reagents to increase sensitivity of the assay. In some cases, the sample pad and the conjugate pad can be the same unit, although this is not common. Typically, the conjugate and the assay buffer are not compatible. However, it is not unusual to see in some assays the same material being used for the sample pad and the conjugate pad, although the pads are treated individually and subsequently assembled.

1.4.1.4 The Wick

Purpose: The wick is the engine of the strip. It is designed to pull all of the fluid added to the strip into this region and to hold it for the duration of the assay. It should not release this fluid back into the assay or false positives can occur.

Material: The material is typically a high-density cellulose. The choice of wicking material is generally dictated by absorptive capacity, cost, and caliper. Tensile strength and availability in rollstock should also be considerations.

Numerous suppliers of these materials are available, with much of the supply coming from Millipore, Whatman, Ahlstrom, and Pall Gelman.

1.4.1.5 Backing Materials

Purpose: All components of the lateral flow assay are laminated to the backing material to provide rigidity and easy handling of the strip. The backing material is coated with a pressure-sensitive adhesive to hold the various components in place.

Material: The backing materials are typically polystyrene or other plastic materials coated with a medium to high tack adhesive.

Considerations: Incorporation of a backing to a lateral flow immunoassay strip is a necessity in order to laminate multiple materials into one unit performing multiple functions. In traditional, non-reader-based lateral flow immunoassays, the lamination process allows relatively large built-in tolerances in component overlaps and final line placement in a cassette. Variations in overlaps can result in variation in run quality of a strip, although in many less demanding applications this variation can be acceptable. In highly demanding applications, however, variations in run time and fluid front conformation can be fatal to the performance of the assay. This is particularly so in reader-based systems, where the evenness of line development across the entire width of the strip, the speed of running to completion, and the position of the developed line in the assembled cassette can all be absolutely critical to the success of the test. This places high demand in precision in the lamination, cutting, and cassette assembly processes. Automation is key to the success of these processes. The use of inline lamination equipment with camera systems, material edge sensing, as well as sensing in cutting and assembly, are all feasible approaches. Consideration should also be given to the tendency of the adhesive to flow into other components of the test strip, notably unbacked membranes, conjugate pads, and sample pads. This can cause disruption of flow patterns and the creation of hydrophobic patches, as well as destabilization of proteins. For this reason, medium, rather than high, tack adhesives are typically used. Care must be taken to choose adhesives that have a track record of being compatible with proteins during storage of the test strips.

Suppliers: G&L, Adhesives Research (Glen Rock, PA). The majority of the market uses G&L backing cards with GL187 pressure-sensitive adhesive.

1.4.1.6 Labels for Detection

The most commonly used particulate detector reagents in lateral flow systems are colloidal gold and monodisperse latex. Latex particles coupled with a variety of detector reagents, such as colored dyes, fluorescent dyes, and magnetic or paramagnetic components, are available commercially. Detailed discussions of these reagents can be found elsewhere in this book (see Chapter 5).

The choice of the particulate label and the detector reagent used in a particular lateral flow system is driven by a variety of factors:

- i) Is covalent attachment of the protein to the particle required?

If this is a requirement, then activated latex particles only can be used, as binding of proteins to colloidal gold is typically achieved via passive absorption.

- ii) Is the assay intended to be quantitative or qualitative? Is a reader required?

The development of truly quantitative lateral flow immunoassays requires a great deal of effort in choosing the basic materials and technologies to be used. Some of these considerations will be discussed later in this chapter. In addition, the integration of readers into these assay systems will also be discussed. If the application requires a reader technology, then the choices of label expand to include colloidal gold, colored or fluorescent latex, and paramagnetic latex particles. The choice of particle and reading technology is driven by the consideration of the cost of the reader technology, the cost and availability of licensing of the readers and the labeling technology, the cost of assay development and reader integration, as well as the performance of the combined reading and labeling technology.

- iii) What are the levels of sensitivity required?

In visually read assays, it is often possible to generate more sensitivity using colloidal gold rather than colored latex particles, due to the smaller size of the gold particles and, as a result, higher packing density can be achieved on a test line. Gold particles are typically in the range of 20–40 nm in size whereas colored latex particles are about 100–300 nm. Gold also has a higher color intensity than colored latex particles, which allows for better discrimination of low positives in an assay. On the other hand, latexes can be produced in multiple colors and can utilize darker colors such as dark blue dyes to provide greater contrast against the white background of a lateral flow membrane. In reader-based assays, it is often possible to generate even higher sensitivity by using fluorescent particles or paramagnetic particles [10, 11].

- iv) Is the assay intended to be multiplexed? Is discrimination between multiple lines of different colors required?

If these parameters are required, the choices are reduced to colored latex particles, which can be produced in multiple colors.

- v) What are the cost considerations in the manufacturing processes?

There are several major suppliers of colloidal gold in the marketplace, such as British Biocell International (BBI, Cardiff) and Diagnostic Consulting Network (DCN, Carlsbad, CA). In general, purchasing colloidal gold is more economical than producing gold internally. It is possible to integrate the process of colloid gold production into the lateral flow immunoassay manufacturing process, and numerous companies have chosen to do this. However, when consistency of the product and the quality of the

assay results are considered important, the overall goal to reduce any sources of variation in the assay often leads companies to contract with dedicated suppliers of high-quality components. There are also multiple sources of the monodisperse latex, most of which are considered to be of high quality, including Bangs Laboratories and Merck/Estapor. The manufacturing of monodisperse latexes is generally not integrated into a lateral flow immunoassay production process. As a result, companies relying on outside supply also have cost considerations.

1.4.2 Processing Methodologies

A comparison of traditional and improved manufacturing technologies will be presented in a separate chapter of this book (see Chapter 8). Only an outline will be given here. The basic processing steps as shown in Fig. 1.4 involve dispensing of reagents, immersion of materials into bulk solutions of reagents, drying of components, lamination of materials, cutting into strips, and packaging. Process designs can be broadly categorized into two methods. In batch processing, card lengths of materials are processed individually, assembled into cards, and cut into strips. In in-line or reel-to-reel processing, all components are maintained in roll format until they have been treated and laminated, and only then are they cut into either individual strip or card lengths for final packaging. It is intuitive and widely supported by industry data [12] that inline processing significantly reduces manufacturing variation in lateral flow immunoassays.

In the final analysis, strip-to-strip variation is among the top concerns expressed by the clinical users of lateral flow technologies (Stratcom, unpublished market research). The reduction of these variations, coupled with the ability to integrate the assays to data collection and reporting systems, will be the key to bringing this technology to a broader range of applications.

1.5 Improving the Utility and Performance of Lateral Flow Immunoassays: Trends in Development of New Technologies

This section considers some of the reasons why much of the potential of rapid membrane testing technology remains untapped, and where this technology may be headed.

The application of lateral flow immunoassays covers multiple market segments with widely diverse performance requirements, market forces, and commercialization strategies. As a result, it is not possible to generate a single definition for the attributes of “next generation” point-of-need assays. However, there are recognized trends and desired design attributes for the development of new tests. Some are met by current designs and approaches to market, while others will require radically different

approaches. Manufacturers looking to develop successful point-of-need products face three challenges. First, they must select and implement features and benefits that cost-effectively match the needs of end users. Second, they must develop the core technologies necessary to create a functional product. And finally, they must ensure that they have selected appropriate market applications for the core technology [13]. The market forces that drive the acceptance of a product in a particular market segment will be discussed elsewhere in this book (see Chapter 8). The remainder of this chapter will concentrate on the discussions of core technology developments considered likely to provide improvement for lateral flow type devices.

1.5.1 Necessary Improvements in Performance Based on Core Assay Technology

Since 1995, over 500 patents have been issued in areas directly related to lateral flow immunoassay technology covering everything from sample pretreatment to improvements in matrices, cassette designs, label types, processing methods, result interpretation, and many others. It is the view of users and developers alike that the technological keys to achieving better market penetration of a point-of-need assay are improvements in test-to-test variation and in sensitivity. Current levels test-to-test variations have prevented the performance of quantitative assays that approach those of larger or more complex clinical analyzers. Improvements in sensitivity would also allow assay systems to be applied in areas where larger clinical immunoassay systems, and methodologies such as PCR, are considered the gold standards. Other general technology features that are considered critical include the integration of reader technologies and data capture systems.

The sensitivity requirement differs significantly depending on the assay and on the application. One consideration is the definition of sensitivity. There are two main definitions applicable to point-of-need assays. The first is “response per unit ligand”. This is the slope of the dose–response curve and is primarily applicable in quantitative assays. The second is “the lowest level of non-zero ligand reliability”. This is measured as the lowest detectable dose (LDD), which is applicable in qualitative assays. Which definition to use depends on the characteristics of the assay. The LDD is the most commonly applied definition in lateral flow immunoassays, where the aim is to distinguish affected from unaffected members of a population. The unit response, however, becomes important when we are considering truly quantitative assays. Both measures are dependent on the slope of the dose–response curve. A steep slope on the curve provides a better unit response and lower LDD than a shallow curve. This in effect defines the discriminatory ability of the assay.

1.5.1.1 Application of Alternative Materials

It is generally accepted that novel materials are needed to improve the functional performance of lateral flow immunoassays. Materials that can perform multiple functions are desirable [11]. The application of new materials opens the door to entirely new concepts of device design and potential levels of performance for LFIA. Some of the preferable characteristics of an improved matrix for point-of-need assays are listed below:

- Highly regular surface, yielding cosmetically high-quality lines
- Three-dimensional matrix with consistent pore size, thickness, and protein-binding capacity
- True capillary flow with a variety of wicking rates
- Thin as reasonably possible
- Good fluid flow characteristics over its entire shelf-life, independent of treatment
- Low CVs for capillary rise time over its entire shelf-life, independent of treatment
- Minimal metal contaminants
- Low background fluorescence
- Non-interfering
- Stable on storage
- Non-flammable
- Low cost
- Can be activated for covalent linkage
- Multiple functionality: can act as conjugate application area, sample application area, reaction surface, separation medium, and wick all in one

Several new materials and approaches that take these basic principles into consideration are under development. Some examples include Fusion 5 from Whatman and the 4CastChip from Amic, Sweden.

i) Fusion 5

One attempt at creating a matrix that meets at least some of the above specifications is the Fusion 5 matrix (Whatman) (see Chapter 7). This is a large-pore, single-layer matrix. It is hydrophilic and non-protein binding in nature. This material fulfills all of the required functionalities of the components in the traditional lateral flow device, namely sample pad, conjugate pad, membrane, and wick. Because the material is non-protein binding, a traditional method of applying the test and control lines onto Fusion 5 is not possible. A strategy of laying down “boulders in the stream” is therefore applied [9]. Large-diameter beads (approximately 2 μm) are used to conjugate to specific proteins and are dispensed onto appropriate locations on the material, where the large-size beads become immobilized and form the test and control line areas. When the test samples and protein conjugates flow past the “boulders”, binding and signal formation occur at those locations. The open pore nature of this system means that assays can be run extremely fast, which have both positive and negative

effects. Speed or reaction time can be important to LFIAs, but can also be related inversely to sensitivity in many instances. With Fusion 5, this is largely overcome due to the lower inherent background and the increased surface area for ligand binding provided by the beads.

The use of Fusion 5 can reduce complexity in the manufacturing process. Multiple dipping, drying, and lamination steps are no longer applicable. The major challenge to the broad application of Fusion 5 as a material is the fact that it comes in only one flow rate, which is very fast. There are no choices in terms of the other material characteristics.

ii) CastChip

A second approach to generate novel assay and material design comes from Amic (Uppsala, Sweden). Amic has developed an assay substrate that consists of a highly ordered array of micropillars on a plastic slide. These micropillars are hydrophilized by dextran, and act to drive capillary flow of sample and reagents in the flowpath. The pillars also provide a biocompatible surface for the attachment of capture ligands at test and control lines. The material is highly regular compared to a standard nitrocellulose material. As with the Fusion 5 approach, the Amic device contains the capacity for multiple functionalities, as the pillar-defined flowpath can act as sample application area, reaction surface, and wick.

Binding of proteins to the surface of the substrate is by covalent attachment via amine linkages to the aldehyde groups on the chip surface. Protein is dispensed onto the surface and then allowed to react in a humid environment for a short time for the linkage to occur. This substrate can generate sensitive assays using fluorescent labels and can be linked to a reader system.

In terms of manufacturing, this device, like the Fusion 5, removes several steps from the process. However, it also introduces the need for discontinuous dispensing of proteins onto discrete substrates, and this can only be achieved in a non-contact manner. From a processing standpoint, the requirement for individual handling of chips is a drawback relative to the ability to handle materials such as nitrocellulose in an inline fashion. Dispensing methods must be highly regular, reproducible, and carefully controlled to ensure that line widths are consistent.

The 4CastChip represents a shift in thinking in the context of LFIA substrates. It is effectively a two-dimensional substrate without discreet pores. However, the device does exhibit true capillary flow; has an extremely regular, hydrophilic surface; and generates cosmetically acceptable lines at sensitivities in systems tested to date that are comparable to existing clinical and POC systems. The material itself is highly stable and maintains proteins in stable conditions for extended periods. As such, it meets many of the criteria previously listed. One challenge to the broad applicability of this system is the cost, which remains high relative to nitrocellulose. Nevertheless, the chip manufacturing technology has the capacity to become very inexpensive with volume, as it is based on CD molding technology.

Fusion 5 and 4CastChip are examples of novel approaches to substrate design, which may or may not ultimately achieve broad market penetration or importance. What is critical, however, is that they represent possible alternatives specifically for application in point-of-need assays. This is a trend that continues to grow, particularly as companies from outside the traditional diagnostic material-supply industries become more interested in this market.

1.5.2 Evolution in Design

It has been argued that the future of the lateral flow technology is inextricably linked to developments in biochip technology (Stratcom, unpublished market research). To some degree, this is true in the broadest sense. In the long term, it is possible that true displacement technology, based on the principles of microfluidics and single-molecule detection, will displace technologies such as the lateral flow device. However, the very characteristics that have defined the broad success of lateral flow – ease of manufacture, low cost and short timeline to development, ease of use and interpretation, broad applicability across sample types and markets, and user and regulatory acceptance – are not inherent in current approaches to biochip and multiplexed and miniaturized assays. The vast majority of these formats still require the development of facilitative technologies for their production and have huge obstacles in terms of proving performance and generating market acceptance. The lessons of i-Stat attempting to introduce new decentralized testing technologies in clinical markets should be carefully studied by those moving into this area. It required years of effort, hundreds of millions of dollars, severe regulatory hurdling, and the development of a whole system of clinical support and distribution to thoroughly break the i-Stat technology into the market. By comparison, up-grading the performance of lateral flow based systems is likely to be much simpler, involving improvements of materials, manufacturing processes, and the application of newer design concepts that are likely to provide faster access to market, with lower costs and burdens.

1.5.2.1 Use of Alternative Conjugate Integration Strategies to Improve Variation Issues

It has been demonstrated that a large component of inter-assay variation derives from the act of impregnating solid substrates with particulate conjugates and drying the conjugates in place before subsequent release using the fluid phase of the assay [14]. As a result, alternative methods for the introduction of conjugate to the assay system can have the result of decreasing assay–assay variation. This has been demonstrated by, for example, Response Biomedical's Rapid Analyte Measurement Platform (RAMP), where the conjugate is premixed with the sample and thereafter delivered directly to the strip along

with the sample. This is one element of RAMP's assay design and result interpretation strategy, particularly the use of the ratio of control to test line intensity ("the RAMP Ratio"), which leads to lower than average strip–strip variation.

1.5.2.2 Integration of Microfluidics to Lateral Flow Systems

Another mechanical element leading to variation in lateral flow immunoassay results is the accurate delivery of sample to the strip and the subsequent movement of the sample and conjugates through the device to the reaction matrix. Several attempts have been made to utilize fluidic elements to overcome the issue of accurate sample delivery. One example is the HemaStrip design, originally developed at Saliva Diagnostic Systems in Vancouver, WA, which utilized a capillary collector molded to the end of a plastic tube in which the strip rests.

1.5.2.3 Sample-Handling Considerations for Assay Sensitivity

For many applications, clinical and otherwise, traditional lateral flow formats and labels are capable of providing significant sensitivity. However, there is a growing trend in certain clinical applications, such as detection of cardiac markers, to attempt to achieve assay sensitivity that would previously have been considered beyond the ability of a point-of-need test to deliver. Other areas that require the same level of extreme sensitivity include biowarfare applications, such as anthrax detection, and food microbiology, where single-organism detection is the goal. Standard approaches of labeling and detection in lateral flow are unlikely to reach the required sensitivities for these applications. Instead, the industry must look to alternate labeling approaches, coupled with the use of reader systems. However, it must be noted that with diagnostic devices, the label and the reader are only part of the equation. The appealing feature of lateral flow and other point-of-need assay systems is that they provide a complete "sample-to-answer" solution in a single step. Simply developing a high-sensitivity reader and labeling system is not enough. It is critical to consider the system as a whole, including the sample, the sampling method, the sample pretreatment methodology, and the concentration of analytes. Analyte concentration can be a confounding factor when it is either too high or too low for detection. There are also considerations of the affinity of the antibodies (see Chapter 4) and how their activities can be affected via the conjugation methods used during labeling procedures. These factors must also be considered when entering into a design program to create highly sensitive and highly reproducible assays. These concepts are illustrated by the following examples:

- i) High concentration of analyte: The high dose hook effect is a well-known phenomenon in assay development. For an immunoassay to give accurate results, there must be an excess of antibodies, both capture and label,

relative to the analyte being detected. Only under conditions of antibody excess does the dose–response curve show a positive slope and provide accurate quantitation. As the concentration of analyte begins to exceed the amount of antibody, the dose–response curve will plateau. Further increase of analyte will cause the slope to become negative. Care must be taken in assay development to validate all potential sample types by dilutional linearity analysis to establish if they are on the positive slope region of the curve. The choice of label, conjugation method, and reading method will all have effects on the ability of the assay to handle large ranges of analyte concentrations. Sample pretreatment may in some cases be necessary to reduce analyte concentration prior to the assay to prevent hook effects.

- ii) Low concentration of analyte: Analytical methods are traditionally divided into several steps: sampling, preliminary operations, measurement, calculation, and evaluation of results [15]. Sampling refers to the generation of a representative sample of an inhomogeneous object [16]. This inhomogeneity presents a challenge to the success of the analytical method. A perfect example of the issues brought about by the quest for the ultimate sensitivity – single-molecule detection – was discussed in an interview with Graham Lidgard of Nanogen published in *IVD Technologies* in May 2006 [17]. Dr. Lidgard took the example of an assay that was developed by Tomas Hirschfeld of the Los Alamos National Laboratory (Los Alamos, NM) [18] in which the researchers coupled a polymer of fluorescein to an antibody and directed a laser through a microscope into the sample. When the molecule passed under the laser, instant photobleaching occurred. The problem was that if there was only a single molecule in solution, it could take three months for this molecule to pass in front of the laser. Therefore, to generate results in a reasonable length of time, several hundred thousand molecules were required to be present in the solution. In a related paper [19], Chen et al. concluded that “the fluctuation in the number of molecules taken for chemical analysis is a fundamental and irreducible source of uncertainty. . . . [and that] the inhomogeneity [of the sampled solution] presents a fundamental limit to analysis”. This same uncertainty applies to the sampling of biological or other matrices for immunodiagnostic testing. As it applies to highly sensitive point-of-need assays, the moral of this tale is that 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 ultimately, it is the concentration of the analyte detectable in the primary sample that is critical. Sampling and pretreatment methods are therefore critical in determining the availability of many analytes in an assay. If one takes 100 ml of a homogenized food sample and concentrates it into 100 μ l for analysis, the assay system will have significantly more sensitivity than one that takes 100 μ l of that primary sample without concentration and tests it. All the sensitivity benefits are there without ever changing a label or a detection method.

- iii) High incidence of cross contaminants in samples (see Chapter 10): Certain assays are well known for their high false positivity rates, including *Mycobacterium tuberculosis* (TB) and *Chlamydia trachomatis*. The Chlamydia assay, where high numbers of organisms are often present, does not require high sensitivity. Rather, it requires antibodies of high specificity. The TB assay has both sensitivity and specificity challenges. The sample pretreatment method, the antibody selection method, and the labeling and reading methods must be designed around these issues.

1.5.3 Reader Systems in Lateral Flow Assays

Reader technologies employed in lateral flow applications (see Chapter 9) are based on one of three labeling and detection technologies: Detection of colloidal gold or colored monodisperse latex particles using charged coupled device (CCD) cameras, detection of fluorescent monodisperse latex particles based on LED excitation via confocal or other optical sensors, and detection of paramagnetic monodisperse latexes using Magnetic Assay Reader (MARTM) technology. It is beyond the scope of this chapter to discuss these technologies in detail, but numerous references are available [10, 11, 23].

The implementation of reader systems in marketed lateral flow systems has been accomplished only sparingly to date. The Biosite Triage and Response Biomedical RAMP systems are among the only examples currently on the market for quantitative applications, and the Biosite device does not utilize a standard lateral flow design. Other readers are used for qualitative assays, including drugs-of-abuse assay systems, such as Cozart's DDSTM or RapiscanTM products, American BioMedica Corporation's RapidReaderTM for their RapidScreenTM, and other drugs-of-abuse assay formats. Non-clinical applications also use visual readers, such as Neogen's Reveal Accuscan, which accepts their lateral flow tests for *Listeria*, *Escherichia coli* O157:H7 and *Salmonella*, and the company's thin test strips for GMOs, mycotoxins, food allergens, and ruminant material. Recently, "in-cassette" readers have been utilized in several products. However, quantification of results is not the intent of these systems either. These devices serve as an analog to digital conversion of a yes/no result, with the purpose of removing user interpretation errors (e.g. the "Clearblue Easy"TM from Inverness Medical).

Several factors can account for the limited application of reader devices in the lateral flow immunoassays:

- i) Most applications of LFIAs have utilized visual labels which do not require readers. Among the many positive attributes of LFIA applications is simplicity. It does not require the cost and complexity of a reader to generate results.

- ii) LFIA's have been traditionally applied in areas where results recording and data capture are not required.
- iii) Quantitative LFIA systems have proven extremely difficult to produce due to variability issues with the assays rather than the readers.
- iv) For reader-integrated device development, access to or development of an appropriate reader system is a major issue. Reader systems are not readily available to developers of lateral flow immunoassays. They are beyond the ability of most lateral flow manufacturers and developers to produce. Limited options for original equipment manufacturers (OEM) of reader systems have traditionally existed. Furthermore, integration of reader systems adds complexity to product design and development over that of traditional LFIA products. Many lateral flow developers do not have the expertise or experience to develop an integrated system and take it through regulatory channels. The cost of such development, even with many of the OEM producers of readers, is inhibitory to most small- and mid-sized lateral flow developers.

Given the fact that readers do not define the ultimate sensitivity of the assay, and that the chemistry and biology ultimately provide the result, where should a developer put the most effort? Clearly, the chemistry and biology of lateral flow immunoassay must be made to work in association with the reader and the sample-handling methodologies. This is the area that provides the most fertile opportunity for assay development companies, the majority of which are unlikely to become directly involved in reader development due to issues of cost and lack of expertise. Several companies provide readers on an OEM basis to the market. The Leach Technology Group manufactures visual reader systems for inclusion in OEM platform products. Members of the Leach Group are Hauser Inc., Westlake Village, CA; UMM Electronics Inc., Indianapolis, IN; and LRE Technology Partner GmbH, Munich and Nordlingen, Germany. The major entrant to the field of OEM reader supply in recent times has been ESE GmbH (Stockach, Germany), with a range of portable benchtop and handheld fluorescent and visual readers that can be integrated with products quickly and cheaply (also see Chapter 9). An example of a fluorescent and visual lateral flow immunoassay reader from ESE is shown in Fig. 1.5. These readers are expected to greatly reduce the complexity and cost of quantitative assay development in the near- to mid-term and should encourage entrants to this field.

It is expected that market requirements will continue to drive this move toward integrated reader technologies for lateral flow immunoassays. Market drivers include the following aspects:

- The effort to push many diagnostic tests out of the central lab into the home and point of need continues. Data capture, appropriate result interpretation with minimal user error, and the opportunity for data mining are all important features of that trend. These requirements will feed the need for reader systems at the point of need, even for qualitative assays. In clinical



Fig. 1.5 ESEQuant portable benchtop lateral flow strip reader by ESE GmbH. Fluorescent or visual labels can be read

applications, integration of the point-of-need assay with the hospital’s information system will be critical to the acceptance and use of the assay by physicians.

- The ability to connect assay results at the point of need with other elements of the healthcare system will make result interpretation, data monitoring and storage, and transduction of data into action feasible at centralized sites with feedback capability.
- Increased sensitivity will require non-visually interpreted results.
- Quantification requires a reader.

1.5.3.1 Consideration of Reader Design for Lateral Flow Applications

Cost, Maintenance, and Calibration: A reader should not be over-engineered. In terms of cost, the consensus among manufacturers in the clinical area is that they will need to give readers away or provide them at low end user cost. So the cost of the reader becomes critical. Abbott established the “razor and blades marketing strategy” in the mid-1980s, with the launch of the first truly automated immunoanalyzer – the TDx. Thereafter, this became the strategy followed by most companies that market tests on small readers (Source: Stratcom). In terms of maintenance requirements, users of readers in point-of-need applications will not tolerate a requirement for regular maintenance or calibration of a reader. As a result, the readers should be simple, robust, and subject to internal or on-strip calibration procedures. This is one strong argument for the

application of single-use devices such as those seen in the Clearblue Easy hCG test (Inverness Medical).

Communication/Connectivity: The reader should be able to collect, store, and communicate data to other sources. The technologies by which this can be accomplished, including Bluetooth and USB, are now standard across multiple industries and relatively simple to implement for reader manufacturers. The requirement for this feature cannot be over-emphasized in any market segment to which the readers will be applied. The human clinical market is a good example. Pressure for having device connectivity comes not only from multiple sources, including physicians themselves, but also from the US government and external industry sources. The US Health and Human Services Office has called for a National Health Network to enable healthcare providers to securely share the myriad of data, records, and images on patients. The aim is to provide seamless healthcare services across systems, doctor's practices, clinics, labs, and hospitals. Compliance to various regulations such as the Health Insurance Portability and Accountability Act (HIPAA) notwithstanding, this effort is estimated to be a \$200 billion dollar market [20]. Major consortia are being formed to address the challenges of this industry, including the Continua Alliance, with such notable members as Cisco Systems, IBM, Oracle, CSC, Microsoft, Hewlett-Packard, and Intel. The presence and interest of companies like these, through organizations like the Continua Alliance, guarantee that connectivity will play an increasingly important role in diagnostics. The integration of reader systems with onboard connectivity into assays will become one of the major trends in point-of-need diagnostics in the twenty-first century.

Throughput: The reader methodology, coupled with how the assay is run, defines the potential throughput of the assay system. Due to the limitations of lateral flow design, and the fact that these are not end-point assays, control of the time at which assays are read can be critical to the generation of appropriate test results in quantitative assays. As a result, certain reader designs used in marketed assays require that the assays be run on the reader, with timing of result reading done by the reader. In essence, that severely limits the throughput of the system. Depending on the application, high throughput may be more or less of an issue; but in applications such as clinical laboratories where tests may be batched before being run, this will be a critical issue. The inclusion of multiple read-heads will be one element that can overcome this concern. However, the ultimate answer lies in the test design and result interpretation algorithms.

Sensitivity: The reader must be calibrated to cover the entire dynamic range of the assay. In certain instances, such as fluorescence, this may require desensitization of the reader relative to potential performance, as available signal may swamp the detector.

Footprint and Portability: Small size and portability may be a positive or negative attribute depending on the market area being addressed. Assuming the unit has onboard connectivity, there is no technological requirement for lateral flow readers to be large, bench-top units. The footprint and size and feel of the reader will be defined by the requirements of different markets.

1.5.4 Examples of Novel Label and Reader Systems for Next-Generation Applications

As discussed previously, the primary labels used in lateral flow immunoassay are colloidal gold and monodisperse latex, labeled with colored, fluorescent, or magnetic tags. The major suppliers of colloidal gold are Diagnostic Consulting Network (DCN) and British Biocell International (BBI). Dyed latexes and paramagnetic particles are available from a variety of sources including Bangs Laboratories, Dynal, Merck/Estapor, and Magsphere. There are also a variety of other labels and enhancement methods that are in development for use in point-of-need assays. One example is nanoparticles. Magnetic, latex, metal, and semiconductor particles on the nanometer scale have unique optical, electronic, and structural properties that can be used in a variety of clinical applications. However, the promised advances in performance based on nanotechnology have yet to materialize in the point-of-need industry. Numerous labeling technologies are in development, but it may yet be many years before some of these technologies produce improvements to existing labeling technologies in real-world applications. As yet, there are few examples of any of these labels in marketed point-of-need assays, particularly in lateral flow format. Examples of newer label technologies can be identified:

- i) Up-converting Phosphor Technology: STC Technologies, Inc. (Bethlehem, PA), now Orasure Technologies, developed the up-converting phosphor technology (UPT). UPT is based on lanthanide-containing submicrometer-sized ceramic particles that can absorb infrared light and emit visible light. They have the advantage that biological matrices do not up-convert. Additionally, the particles do not photobleach and are inert to common assay interferents such as hemoglobin. Orasure Technologies, Inc. developed a reader system based on these particles called the Uplink, and demonstrated its utility in example systems including one for *E. coli* O157: H7 [21]. The label has yet to achieve broad penetration in the market.
- ii) Quantum Dots: Quantum Dot Corporation (Palo Alto, CA), now owned by Invitrogen, originally commercialized the quantum dot (Qdot) nanocrystal technology developed at California's Lawrence Berkeley National Laboratory, the Massachusetts Institute of Technology, and the University of Melbourne (Source: Stratcom). Qdots are 10–20 nm-sized crystals containing a few hundred to a few thousand atoms of cadmium mixed with selenium or tellurium, which have been coated with an additional semiconductor shell (zinc sulfide) to improve the optical properties of the material. The size of the nanocrystal determines the color of the excitation achieved when it is excited with a long-wavelength UV lamp. As a result, multiplexed assays can be created when conjugates of different sized particles are used as labels in the assay system and are excited using a single wavelength [22].

- iii) SERS Tags: Oxonica (Mountain View, CA) produces the Nanoplex biomarker detection system, which utilizes silica-coated, surface-enhanced Raman Scattering (SERS)-active metal nanoparticles as labels for bioconjugates. As with the QDot, the Nanoplex particles boast the ability to produce multiple excitation wavelengths from a single excitation frequency, allowing their use in multiplexed assays [23].

1.6 New and Growing Applications of Lateral Flow Point-of-Need Assays

1.6.1 Point-of-Need Nucleic Acid Tests

Driven by the need to detect nucleic acid products and the opportunity to increase test sensitivity in point-of-need environments, there is a strong market desire to couple nucleic acid detection technology to the speed and convenience of the lateral flow immunoassay. The key to this type of technology lies in the ability to produce highly sensitive results in a very short period of time.

The detection of nucleic acid product in point-of-need environments has been difficult due to a variety of factors. Technical difficulties associated with the most commonly used amplification technology, polymerase chain reaction (PCR), revolve around the challenges of sample preparation, power demands, and control of assay specificity and reproducibility. Complex solutions using automated sample-handling systems coupled with microfluidics and biosensor detection methods are in development, but are not conducive to point-of-need applications. As a result, simpler, alternative strategies are being sought to improve both the amplification and the detection methods. Recombinase Polymerase Amplification (RPA) developed by ASM Scientific (Cambridge, UK) represents a significant step forward in the reduction of the complexity of nucleic acid amplification methods to the point where they can be applied in point-of-need environments [26]. However, lateral flow technologies have not yet been widely applied in this area. A variety of modifications to the standard approach can allow for the application of this technology in Nucleic Acid Lateral Flow (NALF) and be the final step in bringing this key application directly to the market.

A number of strategies are available for the detection of nucleic acids in lateral flow systems [26–29]. The capture of nucleic acids can be performed in an antibody-dependent or antibody-independent way. For example, Piepenburg et al. [27] describe an antibody-dependent system, where an anti-biotin antibody immobilized on the surface of nitrocellulose is used to capture biotin and carboxyfluorescein (FAM) bearing oligonucleotides in RPA amplicons. Binding is subsequently detected using an anti-FAM-colloidal gold conjugate. An antibody-independent alternative utilizes streptavidin as the binding agent. Immobilization of oligonucleotide probes directly onto membranes is

also possible using oligonucleotides linked to carrier proteins. Still another possible configuration is to use the “boulders in the stream” approach, where the oligonucleotides are immobilized onto the surface of large latex beads. The beads are subsequently dispensed onto the reaction matrix, forming the usual test and control lines. This methodology serves to vastly increase the available surface area for binding of analyte, thereby improving the sensitivity of the assay. In terms of detection on a NALF strip, the same labels can be employed as on a standard lateral flow immunoassay, including colloidal gold, colored, and fluorescent monodisperse latex particles. Similarly, the basic elements of the manufacturing and equipment can be readily applied to nucleic acid lateral flow. However, simply applying the basics as currently practiced will also mean applying many of the sources of variation and limitation of the lateral flow technologies. Rational improvements to the basic technologies will be key to unlocking the full potential of this assay format in nucleic acid applications.

1.6.2 Proteomics, Therapeutic Monitoring/Theranomics

The link between proteomics and diagnostics is a logical and potentially critical one. A clear need exists for new diagnostic targets for such pathologies as ovarian cancer, bladder cancer, pancreatic cancer, and Alzheimer’s disease. It is hoped that proteomics will be able to identify such targets. Even though current commercial activities in proteomics are focused on developing analytical technologies, there have been increasing efforts to develop clinical applications as well [30]. An example of the successful use of proteomics coupled with diagnostic tools was illustrated in a paper by Drydna et al. [31]. Using 2D electrophoresis and mass spectrometry, these researchers identified a protein that could differentiate between rheumatoid arthritis and osteoarthritis. An ELISA was subsequently developed for clinical use based on this marker. An important aspect of this work is that it also provided a potential means for monitoring therapy, thereby linking the theranostics concept. By isolating chronic disease markers that are subjected to the influence of therapeutic drugs, the entire loop of discovery, diagnostics, therapeutics, and monitoring is utilized. In this scheme, self-monitoring or doctors’ office monitoring of therapy using rapid assays makes sense on many levels. This is especially so if point-of-need assays can be made to be quantitative and reproducible. The outcome will likely require the utilization of reader and data recording strategies discussed earlier in this chapter. Another example of appropriate application of this strategy is in cardiac diseases. There is currently an active search for coagulation and lipid metabolism proteins that will help improve cardiac risk assessment. In 2005, the world market for these enzyme and protein markers was \$20 million. Most assays are developed for specific drug discovery projects and have not been commercialized on a large scale. Between 2005

and 2010, these tests will gain importance and it is predicted that by 2010 this market segment should grow to \$40 million (Source: Stratcom).

The determination of susceptibility to disease conditions or the probabilities of therapeutic successes is a possible application of rapid tests. In instances where pathologies may have resulted from changes in several proteins, either generative or consequential, diagnostic strategies will require multiplexing and subsequent analysis using algorithms in a process known as profiling [32]. For these applications, lateral flow type assays may not be appropriate as multiplexed systems such as microarrays are more likely to be utilized. However, for simpler systems that rely on the detection of only a single or relatively few proteins, rapid assays based on lateral flow principles may be applied.

1.6.3 Infectious and Chronic Disease

In considering the worldwide market applicability of diagnostics, a socio-economic division is often applied. The world is somewhat arbitrarily divided into First, Second, and Third Worlds, and population, disease states, and diagnostic applications tend to be broadly considered along those lines. This classification ignores the heterogeneity of population and conditions present within each of those arbitrary boundaries. Cardiac and other chronic diseases in the expanding middle classes of emerging economies are growing, as are the incidences of previously geographically limited infectious diseases (e.g., malaria, dengue), emerging diseases (e.g., H5N1 Influenza), and heretofore well-controlled diseases (e.g., TB in First World Countries) in developed countries. Twenty well-known diseases (e.g., cholera, malaria, TB) have reemerged or spread geographically since 1973, often in more virulent and drug-resistant forms. At least 30 previously unknown disease agents have been identified since 1973, including HIV, Ebola, hepatitis C, and SARS (Source: Stratcom). As a result of this globalization of disease states, one of the most critical issues for diagnostic companies is the development of appropriate distribution and sales strategies in worldwide markets, as well as the navigation of local regulatory and legal conditions. In chronic diseases, there remains significant growth particularly in the areas of inflammation, cardiac markers, and cancer, with a myriad of new labels in development in the search for improved diagnostic and prognostic indicators.

1.6.4 Non-human Applications

The application of lateral flow immunoassays beyond the clinical human diagnostics market is also continuing to grow. Examples of areas of applications are listed below:

- i) Animal Health: e.g., equine pregnancy, bovine pregnancy and fertility, and companion animal infectious disease testing.
- ii) Agriculture: e.g., genetically modified organisms (GMO) detection, crop quality testing.
- iii) Biowarfare: anthrax detection
- iv) Environmental and Health and Safety: e.g., contaminating enzymes in manufacturing plants; Legionnaire's disease in air conditioning and water systems.
- v) Food Microbiology: e.g., *E. coli* O157, *Salmonella*, *Listeria*, and other food spoilage organisms.

1.7 Conclusions

Lateral flow immunoassay technology is evolving rapidly. Novel approaches driven by market needs are leading to improvements in performance and utility to a vast array of new application areas. With the integration of new reading, labeling, sample-handling, and device designs comes a requirement for a new approach to system development and manufacturing. The development of highly sensitive and reproducible/quantitative next-generation point-of-need diagnostic assays requires a different, more multidisciplinary approach than has been the case with standard lateral flow immunoassays. Input is required from a range of disciplines, including materials science, chemistry, biology, optics, software and hardware engineering, as well as process design, equipment design, and project management. For this reason, a more collaborative approach is required, and companies such as Diagnostic Consulting Network are established with the purpose of fulfilling the many needs of developers in this complex area.

All of the novel approaches discussed in this chapter, as well as the improvements to standard approaches discussed in another article in this series (see Chapter 8), have ramifications for the developers and manufacturers of LFIA and will require changes in the way we think of LFIA. The acid test for any of these technologies will be market acceptance, which in turn will be driven by

- Relevance to the application and the end user
- Manufacturability of the product
- Cost of the product
- Availability for licensing of the individual technologies, as well as the cost of licensing
- Clear license for use, free of other patent infringement

Careful application of lateral flow technologies in well-chosen market areas, coupled with robust, simple reading technologies, novel materials, the correct labels, modified device designs, and appropriate manufacturing strategies, will drive the acceptance of this technology in a vast array of application areas.

Through continuous improvement and evolution in design and performance, lateral flow principles can be applied in ways that have the potential to create entirely new paradigms in high-sensitivity point-of-need testing and will be applied in the market for a long time to come.

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