EMD MILLIPORE

Rapid Lateral Flow Test Strips

Considerations for Product Development

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Introduction

Lateral flow test strips based on the principles of immunochromatography exist for a wide array of target analytes. The first tests were made for the detection of human chorionic gonadotropin (hCG). Today, there are commercially available tests for monitoring ovulation, detecting infectious disease organisms, analyzing drugs of abuse, and measuring other analytes important to human physiology. Products have also been introduced for veterinary testing, agricultural applications, environmental testing, and product quality evaluation. While the first tests presented qualitative results based on the presence or absence of a signal line, test design has progressed toward semi-quantitative and quantitative assays and the integration of hand-held readers.

Most lateral flow test strips are modeled after existing immunoassay formats. Thus, test strips for hCG are typically sandwich assays; and drugs of abuse are analyzed by competitive or inhibition assays. In serum assays, antibodies are detected as indicators of various disease states. Many variations are possible, but they all have in common the formation of a complex between a detector particle that is free in the sample stream and a capture reagent that is bound to the membrane at the test line (Figure 1).

Taking a lateral flow test strip from the design stage through product development to final manufacturing is a process that employs principles from biology, chemistry, physics and engineering. A test design that works well in the R&D lab is of little use if it is difficult to manufacture reliably in high yield. Similarly, even though a product may work well during in-house testing, if it fails to work reliably in the field, it is unlikely to be successful. Uncharacterized variations in a single material, reagent, or process can be sufficient to ruin consistency of performance.

With these considerations in mind, the purpose of this guide is to provide information on the key aspects of immunochromatographic test strip design, particularly the materials used and their integration with reagents and manufacturing processes. Understanding these principles will help to provide a framework for more rapid test development.

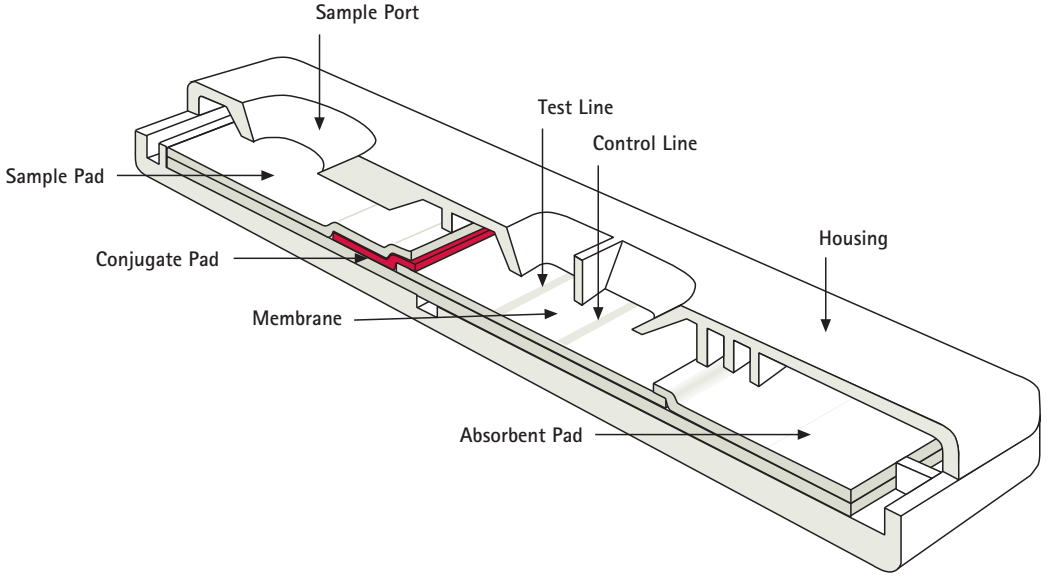


Figure 1.
Schematic view of a lateral flow test strip

Getting Started

Product Description

Before starting test development, it is important to have a basic product design established. There are numerous product qualities that should be determined at the outset so that resources are not spent on a design that isn't marketable. Necessarily, market information will be required to establish certain performance criteria. Key test parameters include: detection limits (sensitivity), required specificity, assay speed, detection format, detection system, test line format, results interpretation, sample matrices, housing design, packaging, labeling, stability requirements, and target cost. It is also critical to understand how much time is available for the test development program.

Antibodies and Other Capture Reagents

Antibodies used in immunochromatographic tests must have sufficient sensitivity, specificity, purity, and stability to accomplish the performance requirements of the finished product. Depending on the assay design, antibodies may be used as a capture reagent at the test line, as a conjugate on the detector particle, or both. As a manufacturing consideration, the antibodies should be available in sufficient supply to meet production forecasts. Purification and consistency of supply are also important. Since the antibodies may be bound to a membrane and detector particle, contaminating proteins will compete for binding sites. There is also the decision to be made as to whether polyclonal or monoclonal antibodies will be used. Minimally, the antibody preparation should be affinity-purified.

A key consideration in antibody selection is the chemical extremes to which the antibodies will be subjected during test strip manufacture and storage. Minimally, the antibody must remain reactive after being adsorbed to a solid surface, retain its structural integrity when completely dried, and then be instantly reactive when rehydrated by the sample. Antibody systems derived from other assays such as ELISA or western blots may not meet these requirements, necessitating screening for alternatives. Also, strategies used to stabilize antibodies in solution are often incompatible with immunochromatography tests.

While formation of an immunocomplex at the test line is most commonly used as the result indicator, it is theoretically possible to achieve a result using any ligand recognition system where a detector particle becomes bridged to a capture reagent on the membrane. In such systems the reagents employed, whether purified from a natural source or prepared as a synthetic construct, are subject to the same requirements for sensitivity and stability as antibodies. It is important to know how these reagents are prepared, what contaminants may be present in the 'purified' product, and what lot-to-lot variations can be anticipated. Purity should not be assumed, but rather demonstrated by the supplier using relevant analytical methods.

Detector Reagents

Various types of detector reagents can be used for the visualization of a signal. The most commonly used materials in commercially available tests are latex beads and colloidal gold particles. Other possibilities include enzyme conjugates, other colloidal metals, fluorescent particles, and magnetic particles. One of the most important features of the particles is that the population is monodisperse with consistency of size and spherical shape. When a test is run, the particles are required to move through the torturous pore structure of the membrane. Smaller particles move faster than larger particles. Particle preparations with different size and shape distributions will move through the membrane differently. This can lead to differences in apparent sensitivity and specificity, even when all other components of the test are identical.

Methods for preparing various particles and conjugating antibodies to them can be found in the literature. Commercial sources are also available. Since the conjugated detector particles are one of the key reagents in the finished test strip, methods for their preparation and handling should be fully validated. Similarly, relevant quality control methods need to be established. If they are purchased from a vendor, they should come with specifications on the attributes relevant to performance in lateral flow tests.

Manufacturing Equipment

Manufacturing schemes range from entirely manual to completely automated. For reproducibility, there are certain steps that require a high level of consistency.

1. Application of reagents onto membranes, sample pads, conjugate pads, and other porous media.
2. Consistent lamination of membranes, sample pads, conjugate pads, and absorbent pads onto a support backing.
3. Precision cutting of sheets or rolls into strips of defined length and width.
4. Assembly of test strips in plastic housings.

Licensing Agreements

There are many patents covering technologies, formats, reagents, and materials that may be of great value in the development of immunochromatographic test devices. Test developers may need to consider licensing one or more of these patents prior to the commercialization of final product. EMD Millipore does not provide legal counsel, nor can it assign rights for any of the patents that have been issued. In the interest of providing useful information to test developers, a list of pertinent patents can be found in the Appendix.

Some patents (e.g., US4855240, US4703017, US4376110 and EP0810436A1) are broadly based and may apply to many different test formats. Other patents are more restricted and may not apply on the basis of reagent selection and test device formatting. Finally, there may be additional patents that pertain to immunochromatographic tests and immunodiagnostic assays in general. To comply with international trade and licensing agreements and to prevent possible legal problems after product launch, it is prudent for test developers and manufacturers to review the patent literature prior to commercialization.

Membrane Selection and Specifications

The membrane is probably the single most important material used in a lateral flow test strip. EMD Millipore manufactures a range of Hi-Flow™ Plus membranes specifically designed for use in this application. Physical and chemical attributes of the membrane affect its capillary flow properties. The capillary flow properties in turn affect reagent deposition, assay sensitivity, assay specificity, and test line consistency. In contrast, other physical properties of the membrane affect integration into finished test strips. This section provides background information on the membrane properties that are important to test strip functionality.

Polymer Composition and Protein Binding

For lateral flow test strips, the membrane must irreversibly bind capture reagents at the test and control lines. The polymer from which the membrane is made determines most of its binding characteristics. If the membrane undergoes a secondary process that chemically alters the polymer or buries it under a second polymer, protein binding properties may be dramatically altered. Typical polymers and their binding properties are presented in Table 1.

Table 1.
Binding properties of different membrane polymers

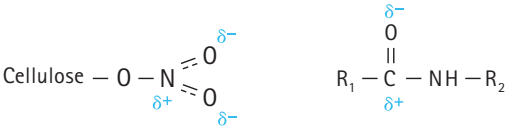
Membrane Polymer	Primary Binding Mechanism
Nitrocellulose	Electrostatic
Polyvinylidene fluoride	Hydrophobic
(Charge-modified) nylon	(Ionic) electrostatic
Polyethersulfone	Hydrophobic

For the most part, a membrane's protein binding capacity is determined by the amount of polymer surface area available for immobilization. The membrane's surface area is determined by pore size, porosity (amount of air in the three dimensional structure), thickness, and, to a minor extent, structural characteristics unique to the polymer. All other parameters being equal, surface area decreases nonlinearly with pore size, increases linearly with thickness, and increases nonlinearly with porosity. For example, the surface area of a 0.1 µm nitrocellulose membrane is about 10 times greater than the surface area of a 10 µm membrane, not 100 times greater. The internal surface area of porous structures is normally reported as m^2/gram of polymer. For membranes, it is relatively easy to determine basis weight in grams/m^2. Multiplying the internal surface area by the basis weight results in the **surface area ratio**, expressed as m^2 internal surface area/m^2 frontal area. For membranes with very small pore sizes (~0.1 µm), this ratio may exceed 2,000. Membranes used in immunochromatographic applications have surface area ratios in the range of 50 to 200.

The loading capacity of a protein on a given surface area depends on the protein's compactness of structure and its Stokes radius (effective diameter). For IgG, the approximate loading capacity is 1 µg/cm^2. Multiplying the loading capacity of IgG (1 µg/cm^2) by the surface area ratio of the membrane (50 – 200) produces an approximate IgG binding capacity of 50 – 200 µg/cm^2. In a typical test strip, the test line is 1 mm wide. If the strip is 1 cm wide, the amount of capture reagent that can be bound is 5 – 20 µg (0.1 cm width x 1 cm long = 0.1 cm^2). This is 10 – 100 times greater than required for most assays. Thus, protein binding capacity is normally not an issue in test design.

Nitrocellulose membranes bind proteins electrostatically through interaction of the strong dipole of the nitrate esters with strong dipoles of the peptide bonds within the protein (Figure 2). Nitrocellulose membranes are completely neutral with no acidic protons. Although their ability to adsorb protein is independent of the pH of the immobilization solution, pH can affect the immobilization efficiency of a particular protein by altering its properties in solution. When applying capture reagents to nitrocellulose membranes, chaotropic agents such as Tween® 20 and Triton™ X-100 should be omitted or used at concentrations < 0.01% v/v. These compounds can physically interfere with molecular contact between the protein and nitrocellulose. If included in the capture reagent buffer during manufacture, the signal may start to develop when the test strip is run and then disappear as the capture reagent lifts off the nitrocellulose and migrates downstream. If chaotropic reagents are required to prevent non-specific interactions or reduce background, they should be put into the sample pad. It is important to recognize that the capture reagent will be

Figure 2.
Structure of nitrocellulose ester and protein dipoles



localized at the test line because it is evaporated in place, but this fact does not imply that the protein is actually adsorbed to the nitrocellulose at the molecular level.

Capillary Flow Rate

The capillary flow rate is the speed at which a sample front moves along a membrane strip when liquid is introduced at one end. This value is very difficult to measure accurately since the rate decays exponentially as the liquid progresses along the membrane. The membrane is in essence functioning as a chromatography matrix. An easier parameter to measure is the capillary flow time, which is the time required for liquid to move along and completely fill a strip of defined length. This value is typically expressed as sec/cm and is inversely related to flow rate, which is expressed as cm/sec. The flow rate of a membrane depends on the aggregate properties of the porous structure. A discussion of these parameters is helpful in understanding capillary flow.

Pore Size

Historically, microporous membranes have been described on the basis of pore size, which is the diameter of the largest pore in the filtration direction, i.e., through the plane of the membrane. Pore size can be determined by challenging the membrane with hard particles and measuring the size of the largest particles that pass through the membrane. Because of the complexity of this technique, it is impractical to implement as a routine QC method. Instead, the parameter measured routinely is bubble point. The bubble point of a membrane is the pressure required to force air through a wet membrane. Although this pressure is somewhat affected by the surface energy of the membrane polymer, the bubble point is predominantly controlled by the diameter of the largest pore. Typically, during the development of a membrane, a correlation is developed between bubble point and pore size (Figure 3).

For membrane evaluation, the bubble point is measured; and the pore size is inferred from the correlation curve. For pore sizes between 0.1 µm and 3 µm, the relationship between bubble point and pore size is fairly linear and reliable. For pore sizes >3 µm, the relationship deteriorates considerably. Consequently, it is inappropriate to rely on pore size as a parameter by which to specify or control the consistency of the highly open membranes used to develop lateral flow test strips. For membranes cast onto a polyester backing, there is the added difficulty that the backing makes it impossible to measure a bubble point.

It is important to understand that the pore size assigned to a filtration membrane is not a measure of the absolute pore size of the structure but rather a description of the functional pore size in filtration applications. As such, it is actually a pore size 'rating.' When microporous membranes are viewed with scanning electron microscopy, measured diameters of the pores are often several times larger than the pore size rating. Examination of lateral flow membranes reveals pores with diameters that exceed 20 µm.

Pore Size Distribution

Whereas pore size is a measure of the diameter of the largest pore for the purpose of particle filtration, pore size distribution reflects the entire range of actual pore sizes throughout the structure. Membranes with identical pore size ratings can have very different pore size distributions. In this case, the pore size rating is far less predictive of capillary flow rate. Ultimately, the capillary flow rate of a lateral flow membrane is determined by the overall distribution of pore sizes. As the aggregate pore size increases, the flow rate of the membrane increases.

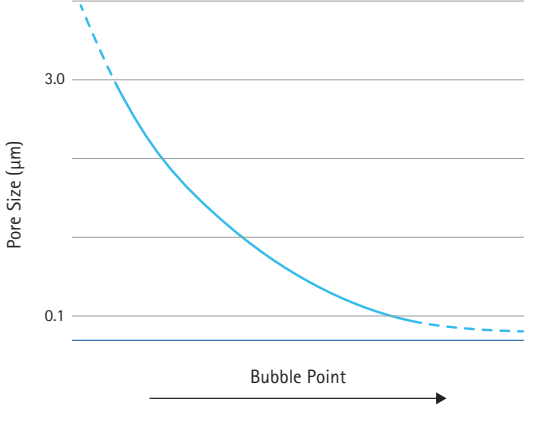


Figure 3.
Relationship between bubble point and pore size

Porosity

Porosity is the volume of air in the three-dimensional membrane structure and is typically given as a percentage of the membrane's total volume. For example, a piece of membrane that measures 1 cm x 1 cm x 0.014 cm (i.e., 140 µm thick) has a volume of 0.014 cm^3 (14 µL). If its porosity is 70%, the volume of air comprising the pores is 9.8 µL (0.7 x 14 µL). The volume of air is also referred to as the membrane's bed volume or void volume. The bed volume of a membrane or other porous material can be used to calculate the total volume of liquid reagent required to wet it out. Porosity is typically unrelated to and uncontrolled by pore size. These two parameters are essentially independent. The same is true for porosity and thickness, although bed volume is directly proportional to both. For EMD Millipore's Hi-Flow™ Plus membranes, the porosities are similar at all capillary flow rates.

This discussion is important because capillary flow rate is the membrane's most critical performance parameter in lateral flow test strips. EMD Millipore was the first membrane manufacturer to manufacture lateral flow membranes and determine product quality on the basis of capillary flow properties. Capillary flow times for Hi-Flow™ Plus membranes are listed in Table 2.

Effects of Capillary Flow Rate on Signal Formation

An absolute requirement for test strip functionality is the development of a detectable signal at the test and control lines. One aspect of lateral flow tests often overlooked is the fact that the system is dynamic. The chemical constituents are constantly moving through the porous materials and never reach equilibrium. Thus, formation of immunocomplexes at the test and control lines depends on the finite time period that the components are sufficiently close at the molecular level to bind to one another. Capillary flow rate determines the length of this time period. The effective concentration of analyte in the sample is inversely proportional to the square of the change in flow rate. For example, if the capillary flow rate increases from X at the test line to 1.25X, the effective concentration of analyte decreases by 36% (1-(1/1.25^2)) (Figure 4).

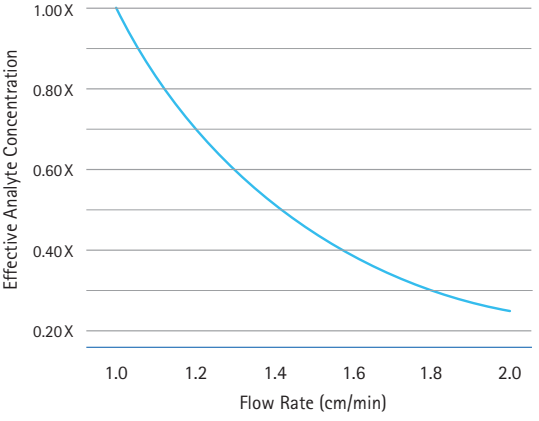
In a lateral flow test strip, the antigen (Ag) is unable to bind once its passes the immobilized antibody (Ab) because test strips are designed to flow in only one direction. As a consequence of this strip property, the effective Ag concentration decreases with the square of the increase in flow rate because of the reduced length of time that the components of the reactive pair are close enough to bind to each other. The amount of complex formed, R, is equal

Table 2.
Capillary flow times for Hi-Flow™ Plus Membranes

Membrane	Capillary Flow Time Specification* (sec/4 cm)	Flow Rate	Sensitivity
HF180	180 ± 45	Slowest	Most sensitive
HF135	135 ± 34	↓	↓
HF120	120 ± 30		
HF090	90 ± 23		
HF075	75 ± 19	Fastest	Least sensitive

*The range is for all measured values on a roll and represents ± 3 ò; the acceptable range for the mean is ± 10% of the target.

Figure 4.
Effect of capillary flow rate on assay sensitivity



Examples:

Flow Rate = 1.00 cm/min → Effective Analyte Concentration = 1.00 X
Flow Rate = 1.25 cm/min → Effective Analyte Concentration = 0.65 X

to k, a rate constant related to the affinity of the antibody for the antigen, times the concentrations of the reactants Ab and Ag. At a flow rate of 1X:

$$R = k[Ab][Ag]$$

If the flow rate doubles, each component is only close enough for half the amount of time. [Ab] and [Ag] don't actually change; their *effective* concentrations change because they spend only half as much time in close enough proximity to bind to each other. At a flow rate of 2X:

$$R = k[0.5 \times Ab][0.5 \times Ag] = 0.25\ k[Ab][Ag]$$

The impact on the amount of complex formed is equivalent to reducing the concentration of Ag by a factor of 4 compared to the original flow rate. This relationship is the principal reason why it is often impossible to use the fastest lateral flow membranes. Moving from a membrane with a flow time of 180 seconds/4 cm (e.g., HF180) to a membrane with a flow time of 90 seconds/4 cm (e.g., HF090) would result in a four-fold decrease in assay sensitivity. In some cases, it is possible to compensate for the sensitivity loss by increasing capture reagent and detector particle concentrations. This compensation has its own drawbacks: increased reagent cost, loss of specificity, and high assay background. Also, the analyte concentration eventually becomes limiting.

Another important consideration is the fact that the capillary flow rate decreases exponentially as the distance of the sample front from the origin increases. Typically, it takes twice as long for the flow front to move from X cm to 2X cm as it takes to move from 0 cm to X cm. In other words, if it takes 1 minute to travel 1 cm, it will take 2 more minutes to travel the next centimeter. The implication of this phenomenon is that the placement of the test line has a significant impact on achievable sensitivity. As the capture line is placed further from the origin, the flow rate at which the analyte passes the capture reagent line is slower; and the effective concentration of analyte in the sample is higher. For this reason, it would seem to be advantageous to locate the capture line(s) relatively far up the strip. This is generally the case when an absorbent pad is used (Figure 1) because the pad's bed volume controls the volume of sample that flows past the capture reagent line.

When an absorbent pad is not used, the volume of sample that flows past the capture line depends on the capture line's position. As the capture line is moved further from the origin, the volume of sample that can be analyzed and the quantity of detector reagent/analyte complex that can be captured both decrease. The improved kinetics obtained by locating the line further from the origin needs to be balanced against the reduction in the amount of sample analyzed.

Finally, there may be constraints imposed by the plastic housing. If the housing design is fixed early during test development, it may be difficult to reposition viewing ports because of financial ramifications associated with new molds and delayed production. In this instance the line positions can only be moved within the range that allows the signal to remain visible.

Since the flow rate of the analyte past a capture reagent determines its effective concentration, it should be obvious that the kinetics of binding at the test and control lines are different because of their separation on the membrane. Similarly, in a multiplex assay, the reaction kinetics at each test line will vary. The amount of capture reagent and detector particle for the first test line may need to be increased to compensate for the fact that the sample will flow more rapidly past this line than one farther downstream.

There is a third way that capillary flow rate affects overall assay performance. As the capillary flow rate increases, a given volume of liquid spreads out further on the membrane, other physical properties being constant. This is a critical point to consider when optimizing protocols for striping reagents onto the membrane. Reagent lines applied to faster flowing membranes will be wider than the same lines applied to slower flowing membranes. This causes the signal to be spread out over a wider area, making it more difficult to visualize a weak signal. Specifications for sensitivity are often established at analyte concentrations that produce weak signals, so that variation in line width can adversely affect assessment of performance at the limit of sensitivity. A wider line may be below the threshold of visibility.

Changing the Dimensions of the Membrane to Control Sensitivity

If no absorbent pad is included, the volume of sample that can be analyzed depends on the bed volume of the membrane. Consequently, there may be considerable benefit derived from changing the membrane dimensions (length and width), as illustrated in Figure 5.

In both cases the area of the membrane strips is approximately 5 cm^2, and the capture reagent line is located 2 cm from the origin. The sample flow rate at the capture reagent line in both strips is equal even though the time required to run example B to completion would be longer. Relative to viewing the test results, the width of the strip is unimportant; both lines can be easily seen. More importantly, example B has 17% more bed volume downstream of the capture reagent line than strip A. This means that 17% more sample passes over the capture line in example B; and the achievable assay sensitivity for example B could be proportionally higher.

Once again, changing the dimensions of the membrane strip to control sensitivity is irrelevant if there is an absorbent pad, as the volume of sample analyzed will be controlled by the pad's bed volume.

Thickness

Membrane thickness is important for four reasons: impact on bed volume; impact on the width of the test and control lines; impact on tensile strength; and impact on signal visibility.

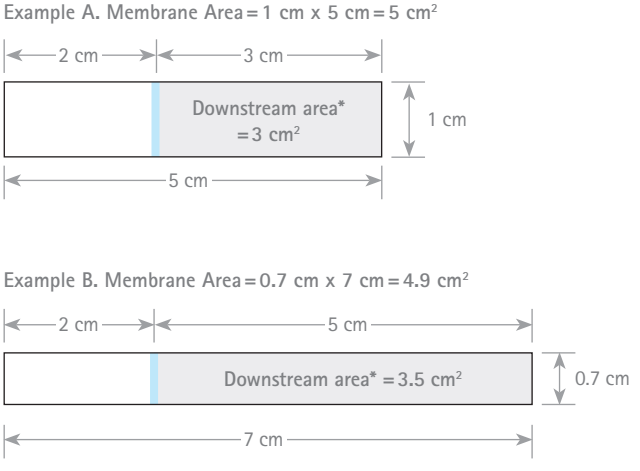
Impact on Bed Volume

Bed volume is directly related to thickness. As thickness increases, the bed volume increases, since the three dimensional space equals the length of the strip times the width times the thickness. For a strip measuring 1 cm wide by 5 cm long by 130 µm (0.013 cm) thick, the total volume equals 0.065 cm^3. If the porosity of the membrane is 70%, the bed volume equals 45.5 µL (0.7 x 65 µL). If the thickness is lowered to 110 µm, the bed volume decreases to 38.5 µL. Conversely, if the thickness increases to 150 µm, the bed volume increases to 52.5 µL. If the total volume of sample to be analyzed is controlled by the membrane's bed volume, the change from 52.5 µL to 38.5 µL, a decrease of 27%, is significant. The bed volume consideration may be unimportant if the strip design calls for the use of an absorbent pad at the end of the membrane strip (see Figure 1). Under these circumstances, the total volume of sample taken up by the strip is governed mainly by the bed volume of the absorbent pad. Also, the volume of sample analyzed may not be proportional to the volume of sample that contributes to the test signal (sensitivity).

Impact on the Width of the Test and Control Lines

Since bed volume increases with thickness, the width of the test and control lines can be affected. In most instances the capture reagents are applied to the membrane at a constant volume per unit distance. The liquid is absorbed into the membrane, displacing the air from a comparable volume within the pores. The liquid usually penetrates down into the membrane and then moves laterally.

Figure 5.
Effect of membrane dimensions on volume of sample analyzed



Spreading of the reagent on relatively thinner membranes will be greater than on relatively thicker membranes because there is less depth to allow downward penetration of the liquid. If the same mass of capture reagent is spread out over a wider area, the detector reagent will be similarly diffused, the net result being less color intensity and lower sensitivity.

Impact on Tensile Strength

Tensile strength, the force at which a membrane breaks, decreases proportionately with decreasing thickness and with increasing pore size. Membranes for lateral flow test strips are very open. If they are not cast onto a nonporous film or web-supported, they are extremely weak. Typical tensile strengths for lateral flow membranes range from 1 to 5 pounds/inch, which is equivalent to 1.75 to 8.8 Newtons/cm. The brittleness of these membranes makes them difficult to handle without breaking, especially when used as 100-meter rolls slit to 2.5 cm wide. For this reason, lowering the thickness of an unbacked or unsupported membrane below 100 µm is impractical. Backed membranes have good tensile strength and are virtually impossible to break during routine processing because of the strength of the film.

Impact on Signal Visibility

Thicker membranes absorb more sample volume, thereby increasing the mass of analyte that passes by the capture line. The signal should also be intrinsically stronger. Both of these potential advantages are negated by a physical limitation of the membrane. Because of the opacity of the nitrocellulose, detector particles bound at the test and control lines are visible only if they are located near the surfaces of the membrane (Figure 6).

Analyte and detector reagent flow evenly throughout the entire thickness of the membrane. In most reagent application protocols, capture reagent also ends up being evenly distributed throughout the thickness of the membrane. In some cases, the antibody may be more concentrated on the side to which it is applied; but for the most part, no discernible gradient is observed. Consequently, detector reagent is captured evenly through the depth of the membrane. Relative to visualization, however, any detector reagent bound deeper than ~10 µm from the surface becomes invisible to the user, as its color is masked by the membrane's opacity.

The percent of visible signal is therefore related to the membrane's thickness:

$$\% \text{ Visible Signal} = \frac{\text{Visible Depth } (\sim 10\ \mu m)}{\text{Membrane Thickness}}$$

Since the visible depth is constant for a given membrane, the amount of visible signal is inversely proportional to membrane thickness. This negative impact of thickness on sensitivity is relatively minor (generally less than 10%), but it may be enough to offset any gain obtained by increasing the membrane's bed volume. This limitation on visibility is irrelevant for detection using magnetic particles, where the detector reads through the entire depth of the membrane. Instead, consistent thickness is very important as the distribution of particles in the magnetic field affects the magnitude of the signal.

Backing

Nitrocellulose membranes manufactured for lateral flow test strips exist in backed and unbacked forms. Backed membranes cast onto a nonporous, polyester film offer two advantages. First, they are easier to handle. The film

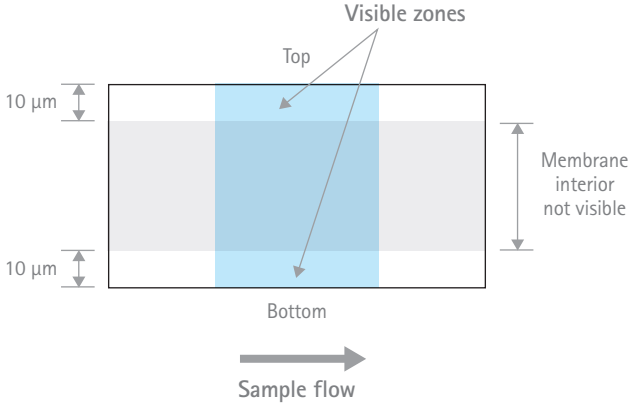


Figure 6.
Cross-section of the capture line

provides a high level of mechanical strength, making it nearly impossible to break the membrane during routine processing operations. Unbacked membranes, in comparison, are extremely fragile and can be very difficult to work with during test strip assembly. Second, the film acts as a barrier to chemical contamination from adhesives that are used to mount the membrane on cards during test strip manufacture. Although adhesives typically do not penetrate into a microporous membrane more than a few microns, volatile organic solvents released from the adhesive during long-term storage may adsorb to the nitrocellulose. This can alter the membrane's wetting properties and adversely affect lateral flow properties. For these reasons, most lateral flow tests are produced using backed membranes.

In today's market, the most common backings are 2-mil and 4-mil polyester films. Membranes on these backings perform similarly. Film thickness is dictated by constraints of the test strip manufacturing process and the tolerances of the intended space in the housing. If the film is too thick for the manufacturing process or the internal features of the housing, the membrane may be crushed and its performance compromised. It is important to note that membranes on a 2-mil backing are assigned the same hazard classification as unbacked membranes (UN3270) and must be shipped under the same standards, often at additional cost. Membranes on a 4-mil backing do not have this requirement.

For unbacked membranes the polymer solution is cast directly onto a stainless steel belt. At the end of the manufacturing process, the membrane is pulled off the belt and wound into rolls. The membrane is porous on both sides, and liquid reagents can be applied to either side. The belt side generally has superior surface quality. The disparity in surface quality between the belt side and the air side generally becomes more apparent as pore size and flow rate increase. The belt side is typically smoother (shinier) and rarely has physical irregularities. For these reasons, it is usually preferable to apply reagents to the belt side of unbacked membranes.

When the membrane is cast onto a plastic film, the belt side is masked, requiring that reagents be applied to the air side. In choosing the membrane for a particular test, it is prudent to understand the types and degree of surface variations that can be anticipated on the air side.

Detergents and Surfactants

Membranes made from nitrocellulose and nitrocellulose/cellulose acetate blends are naturally hydrophobic. They wet easily in water because detergents or surfactants have been added to the membrane during production. The type and amount used are normally fixed by the membrane manufacturer for compatibility with the casting process and broad range compatibility with lateral flow assays. There are four key points to consider when using detergents in manufacturing protocols.

Impact on Capillary Flow Rate

A minimum concentration of surfactant or detergent is needed to make the membrane wettable. Once this concentration is reached, further increases have little impact on wettability. Typically, however, a somewhat higher concentration is required for uniform capillary flow. As already mentioned above, the manufacturer fixes the concentration in the membrane during production.

Impact on Protein Adsorption

Surfactants and detergents can affect protein adsorption, whether derived from the membrane or included in the reagent buffer. If their concentration in the membrane is too high, they will prevent adsorption by masking the nitrocellulose. Manufacturers avoid this during membrane production.

Normally, though, surfactants and detergents are more problematic when included in the reagent buffer. Tween® 20, Triton™ X-100, glycerin, polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), and polyethylene glycol (PEG) can reduce protein binding by adsorbing to the membrane preferentially or forming a complex with a protein molecule before it adsorbs. This is especially true if the critical micelle concentration of a surfactant is exceeded. Their use should be minimized or avoided completely until after the capture reagents have been immobilized and fixed. If they are applied to the membrane as part of a blocking process, their concentrations should be kept as low as possible to prevent displacement of the capture reagent ($<0.05\%$ v/v for Tween® 20 and Triton™ X-100, $<0.1\%$ v/v for glycerin, $<0.5\%$ w/v for PVA, PVP, and PEG). Ionic detergents, such as sodium dodecylsulfate, are extremely effective wetting agents at concentrations well below those that decrease protein binding to the membrane.

Impact on Reagent Striping

When striping reagents onto the membrane, the ease and consistency with which reagent solutions enter the membrane are affected by its wettability. Increasing the concentration of detergent in the membrane above the minimal concentration needed for wettability enhances the consistency of reagent application just as its improves the consistency of lateral flow. At the optimal detergent concentration, the line width should be consistent as long as the application protocol and environmental conditions are optimized and held constant.

Impact on Band Width

Once a minimum detergent concentration is reached, the membrane wets consistently; and the quality of the striped line is maximized. There is also an upper limit that controls the extent of reagent spreading on the membrane. As the amount of detergent or surfactant in the membrane increases, the applied reagent band becomes broader. Although the protein in the reagent solution may not spread as far as the liquid, there is still a proportional impact. To the extent that spreading occurs, there can be a loss in signal intensity and a potential decrease in overall assay sensitivity. For this reason, manufacturers take care not to exceed the optimal concentration of wetting agents. The effects of detergent or surfactant concentration on capillary flow rate, protein binding, striping consistency, and stripe width are summarized in Figure 7.

Maintaining Wettability by Adding Detergents and Surfactants to Solutions Used during Reagent Application and Blocking

The application of liquid reagents, especially blocking solutions, may remove surfactants and detergents from the membrane, or lower their concentrations, and change overall wetting characteristics. The easiest way to avoid this is to add a low concentration of surfactant or detergent (typically 0.01% – 0.05% v/v or w/v) into all or some of the applied solutions, especially the blocking buffer or final wash buffer.

Pore Filling as a Function of Capillary Flow Rate

The efficiency of pore filling depends on the average pore diameter. As pore diameter increases, the pores fill less efficiently. This relationship is driven by capillary pressure, the same force that controls the relationship between pore diameter and capillary flow rate. The implication of this relationship is that a given volume of liquid spreads further on a faster flowing membrane than on a slower flowing membrane, assuming constant porosity. This is unavoidable and must be considered when evaluating the impact of flow rate on assay performance.

Liquid Spreading vs. Protein Spreading

For optimal assay performance, the width of the capture reagent line at test development is far more important than the width of the liquid line at striping. Signal intensity increases with decreasing reagent line width because the same amount of detector particle is focused in a narrower area. In most cases, the width of the capture reagent line is narrower than the liquid line width. Protein molecules adsorb nearly instantaneously when they first contact nitrocellulose polymer. The net result is that they are pulled out of solution by the membrane. Protein molecules will migrate laterally in the membrane if the nitrocellulose is already saturated or if the protein molecules are chemically masked. Unless the protein is added at a saturating concentration,* the width of the protein line is determined primarily by the contact zone between the dispensed liquid and the membrane. This is in turn related to the contact

* Using IgG as an example, if the protein concentration in the reagent solution approaches the saturation level of the membrane (~5 mg/mL IgG), then the width of the capture reagent and liquid lines will be the same. The saturation concentration for a membrane can be determined by dividing the membrane's protein binding capacity (µg IgG/cm²) by the membrane's bed volume (µL/cm²). As stated in earlier sections, the protein binding capacities for this type of nitrocellulose membrane range between 50 and 200 µg IgG/cm². Typical void volumes are approximately 13 µL/cm². Saturating protein concentrations are therefore in the range of 50 – 200 µg IgG/13 µL, or 4 – 15 mg/mL. Antibody solutions rarely approach even the low end of this concentration range.

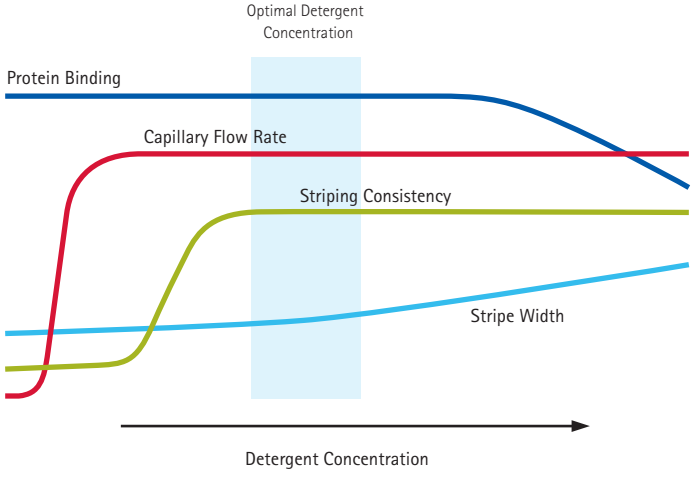


Figure 7.
Effect of detergent or surfactant concentration on various membrane performance characteristics

angle between the liquid stream and the membrane surface. As the contact zone becomes narrower, the protein line becomes narrower (Figure 8).

To some extent, the capillary flow rate of the membrane affects band spreading. As the flow rate increases, the liquid line widens. Consequently, the capture reagent line widens. Potentially, this effect can be overcome by modifying the reagent buffer and/or decreasing the detergent concentration in the membrane.

When capture reagent solutions are striped onto membranes, they penetrate laterally and downward, ultimately filling the space defined by the volume of liquid applied (expressed as µL/linear cm), the bed volume of the membrane, and the efficiency of pore filling. For example, if the dispensing rate is 1 µL/cm, the bed volume of the membrane is 10 µL/cm^2, and the pores fill completely, then the calculated band width would be 0.1 cm (Figure 9).

If the reagent solution penetrates into the membrane slowly due to marginal membrane wettability, then the solution will also tend to be absorbed unevenly. In this case, the reagent line width may differ from the calculated value. In most instances, this is accompanied by variable line quality, which ultimately affects the quality of the signal.

Recommended Storage Conditions (Hi-Flow™ Plus Membranes)

Nitrocellulose membranes do not require specialized storage conditions; however, they should be protected from exposure to organic solvent vapors. Organic solvents adsorb to nitrocellulose membranes and can cause them to become hydrophobic. EMD Millipore recommends that its membranes remain sealed in their packaging to prevent penetration of dirt and dust. For long-term storage, the temperature should range from 10 to 25 °C. The relative humidity should range from 30 to 70%, and the membranes should not be exposed to a condensing atmosphere. Precautions against fire must be implemented as nitrocellulose is extremely flammable. Nitrocellulose membranes are typically greater than 70% air by volume and made from polymers that contain 12% nitrogen by weight. As such, they are only highly flammable, not explosive. At minimum, no flames or open lights should be permitted in the storage area; and appropriate fire extinguishers should be easily accessible. Also, backed membranes do not burn at the speed of unbacked membranes.

It is important to point out that the conditions of long term membrane storage are different from the precise temperature and relative humidity control required in

Figure 8.
Protein band width as a function of liquid contact zone

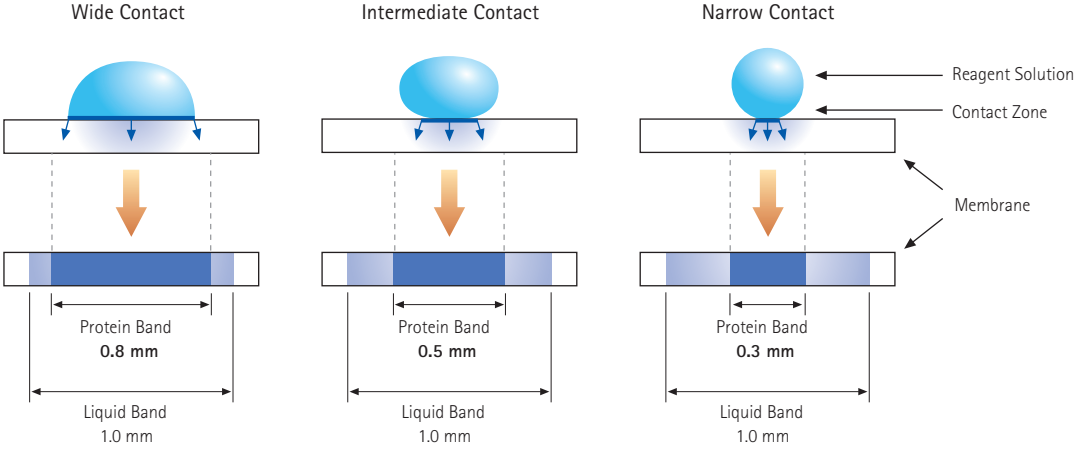
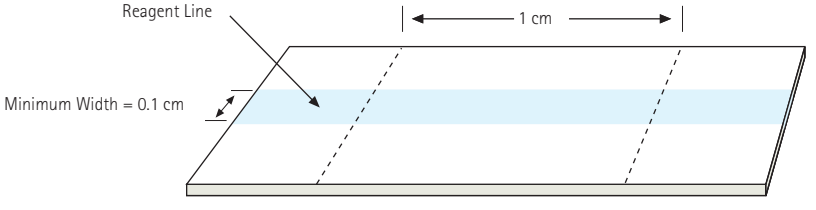


Figure 9.
Calculation of band width as a function of dispense rate



Membrane Bed Volume = 10 µL/cm^2

Reagent Dispense Rate = 1 µL/cm

$$\text{Band Width} = \frac{\text{Reagent Dispense Rate}}{\text{Membrane Bed Volume}} = \frac{1\ \mu L/cm}{10\ \mu L/cm^2} = 0.1\ cm$$

the manufacturing areas. Also, the membrane stability profile is not predictive of test strip stability. Typically, the biological components of the test device determine overall product stability.

Specifying the Membrane

At the end of the product development process, membrane parameters identified as having an impact on final product performance need to be precisely specified. Ideally, each specification is verified by testing at least three lots of membrane made to the specification. Whenever possible, all critical specification ranges should be confirmed by evaluating the performance of membrane limit samples. Limit samples are rolls of membrane that have been deliberately made at the extremes of a specification range. If the specification range has been correctly defined, the membrane limit samples should work acceptably in the final product. If a limit sample does not work, the specification range may be too broad and will have to be adjusted so that it does not include values represented by the failed sample(s).

Capillary Flow Time (Mean and Variation)

The capillary flow time is the most important specification and is expressed by EMD Millipore as **sec/4 cm**. In principle the distance can correspond to the length of the membrane strip in the final assay. This parameter is normally specified as a mean with minimum and maximum acceptable values for individual points. The solution used to perform the capillary flow test must be defined and used consistently during QC evaluation. EMD Millipore uses water (18.2 Ω Ohm resistivity) for routine testing. Humidity and temperature in the testing room must also be controlled because they affect the rate that water evaporates from the exposed surface of the membrane. Standard testing conditions used by EMD Millipore are 45 – 55% relative humidity and 19.5 – 22.5 °C.

Thickness (Mean and Variation)

Thickness is typically given in microns (µm). This parameter is normally specified as an average with minimum and maximum acceptable values for individual points. The thickness of the plastic backing, if present, should be specified separately. For backed membranes, the total thickness (membrane + backing) is reported.

Tensile Strength

Tensile strength is normally measured in pounds/inch (lb/in). The conversion factor for the metric equivalent, Newtons/centimeter (N/cm), is 1.75 (i.e., 1 lb/in = 1.75 N/cm). This is an extremely important specification for unbacked membranes that are slit to narrow widths. The tensile strength of nitrocellulose membranes used for this application is in the range of 1 to 5 lbs/in. If the tensile strength of the membrane is < 1 lb/in, and the product is being used as a 1-inch wide roll, then the equipment designed to process the membrane must operate at a tension of < 1 lb total. Tensile strength is normally not an issue with backed membranes.

Surface Quality

The surface quality of the membrane encompasses many different visual characteristics, some of which will be explained in subsequent sections. It is important to note that many visual features appear on only one side of the membrane. Some of these characteristics vary in their frequency and severity, so that their quantification becomes extremely difficult. One of the best strategies for developing a surface quality specification is to use a library of graded limit samples as the basis for acceptance or rejection of membrane. In order for this to work, the membrane manufacturer needs to produce samples of membrane that have increasing frequency and severity of visual defects. The test strip developer then needs to manufacture final product with each of these samples to determine at what point the quality of final product becomes unacceptable. These results are discussed with the membrane manufacturer; and the surface quality of the worst, acceptable membrane sample becomes the specification limit. Reference libraries of graded samples are retained by the membrane manufacturer and test strip developer to ensure quality standards are maintained.

Powder and Dust (Air Side Only)

Because of the way most nitrocellulose membranes are made, some of the nitrocellulose polymer occasionally remains unincorporated into the membrane structure. When this occurs, the unincorporated nitrocellulose appears as powder on the membrane's air side. The powder can become dislodged from the membrane during test strip manufacturing, appearing as dust on various surfaces of manufacturing equipment. EMD Millipore's Hi-Flow™ Plus membranes are normally free of powder. As the capillary flow rate increases, the likelihood and severity of powder occurrence increase. High levels of powder can make it difficult to apply reagents to the air side or to laminate to the air side using adhesives. Typically, most manufacturing steps will tolerate some level of dust. The problem is defining the upper limit of acceptability since powder can not be readily quantified.

Stucco (Air Side Only)

Stucco is a term used to describe areas of the membrane's air side that are rough like stucco or sandpaper. EMD Millipore's Hi-Flow™ Plus membranes are typically free of this defect. Stucco is very similar to dust in its cause and occurrence, and is easily detected by visual examination of the membrane. In severe cases, the roughness can be felt by rubbing a finger over the membrane. Membrane areas that are affected by stucco will generally not have dust. The occurrence and severity of stucco may vary within

a membrane roll. Stucco causes the same manufacturing problems as dust. When developing a specification for stucco, it is useful to identify the acceptable limit for the worst areas.

Casting Lines

Casting lines relate to how the nitrocellulose lacquer spreads on the belt at the beginning of the casting process. Unevenness in the film results in the formation of casting lines. If they are severe, they may interfere with the flow properties of the membrane. For this reason they should be evaluated and specified.

Pinholes

Pinholes are small holes (≤0.5 mm) in the membrane caused by tiny air bubbles in the polymer solution. When these occur in filtration products, the membrane is automatically rejected. Unless the frequency is very high, pinholes do not normally have a deleterious effect on lateral flow membranes.

Scratches, Dents

During manufacturing, slitting, or packaging, the membrane may be scratched or dented due to physical contact. These defects affect the continuity of flow and can cause non-specific trapping of detector particles or block flow in extreme cases. If the frequency is high enough, they may be cause for rejection of a lot. If the lot is accepted, affected areas should not be used for test strip production.

Orange Skin

Orange skin is a term that is used to define several abnormalities in the membrane's surface. Unlike the visual characteristics that have already been discussed, orange skin should be completely unacceptable under all circumstances. This defect is caused by problems in the polymer solution that result in dramatic differences in the porosity of the membrane from millimeter to millimeter. The mottled white and off white appearance of the membrane looks somewhat like the surface of an orange, even though the defect is inconsistent with either variation in thickness or increase in surface roughness.

Particles

All membranes are slit into rolls or die-cut for conversion to sheets. In a cutting operation, small shards of nitrocellulose are often generated. Because of static interactions, these particles can stick to the slit roll and become trapped between wraps, denting the membrane or interfering with reagent application and lamination. Particles can also be generated during test strip manufacture. This defect is sometimes confused with powder because of the similar size of the particles. They should not be confused as they are corrected in different ways.

Color

Nitrocellulose membranes should be white with no tinge of any hue. Instrumentation used in the paint and fabric industries to measure color can be used to quantify acceptable limits on color. Membranes may discolor from exposure to organic solvent vapors. Due to the very open pore structure of membranes used for immunochromatography, they may appear translucent, especially when wet.

Dirt, Grease, and Foreign Matter

During the process of manufacturing, quality control, slitting, packaging, and transport, membranes come into contact with many different surfaces and are handled by numerous people. Consequently, there is a risk that the membrane will be contaminated. If contamination can be tolerated at all, it should be limited to the outermost wraps of rolls or to the top and bottom sheets of boxes.

Dimensions (Mean and Variation)

The means of the length and width and their tolerances should be defined.

Packaging, Certification, and Labeling

Because of ISO standards, GMP practices, and other regulations, membrane manufacturers are usually well versed in the basic requirements for packaging, labeling, and certification. Packaging specifications for membrane rolls include core dimensions, core materials, end caps, interleaf material, splice limitations, addition of leader or tail sections, outer wrapping, desiccants, the composition and grade of packaging materials, the size and composition of shipping boxes, the number of rolls per box, and other packing materials. Packaging of membrane sheets is less complicated. Important specifications include number of sheets per package, interleaf material, wrapping, desiccants, composition and grade of packaging, the dimensions and composition of the inner (product) boxes, the size and composition of shipping boxes, the number of product boxes per shipping box, and other packing materials.

Basic information for labeling includes product name, catalog number, lot number, and dimensions. Placement of the label on individual packages and shipping boxes should also be specified.

The content and format of the **Certificate of Assurance** or **Compliance** should be detailed thoroughly. The Quality Assurance manager should work with the membrane manufacturer to develop a product certification that meets the quality and regulatory requirements of the final product.

Manufacturers are usually able to customize the packaging, labeling, and certification. Bear in mind that significant deviations from the manufacturer's existing standards can result in higher cost of the product.

Other

There are specifications that will be unique for each application. Some of these might include sampling plans, product retain policies, notification policies, unique release test parameters, and minimum lot size. To the extent possible, these requirements should be identified and agreed to during the specification process. There should be a willingness on the part of both the test strip manufacturer and the membrane manufacturer to modify or add a specification when it becomes clear that, by making the change, the quality of the membrane is enhanced.

Pad Materials

Pad materials comprise the porous matrices that are used for the sample pad, conjugate pad, and absorbent pad. Most commonly, cellulosic materials (i.e., filter papers) are used for sample and absorbent pads, while glass fiber filters are used for the conjugate pad. EMD Millipore offers a range of cellulosic and glass fiber materials under the SureWick® name (Table 3).

These materials are typically much cheaper than nitrocellulose membranes as they are easier to manufacture. In most cases, however, these materials are not being manufactured specifically for utilization in lateral flow tests. Depending on where pad materials are sourced, they may lack specifications that pertain to lateral flow tests and exhibit levels of variability that are greater than desired, particularly at the dimensions that they are being used in lateral flow tests. Other materials, such as woven meshes and synthetic nonwovens, are also used as pad materials, although at a much lower frequency. The functions of the pads and their specifications are discussed below.

Chemistries on Pad Materials

The sample and conjugate pad serve as the location for chemicals that are essential to the running of the lateral flow test. Specific details are described below for each type of pad. There is one important aspect of these chemicals that is generally unappreciated. The chemicals are loaded into these pads by applying homogenous solutions and evaporating the water. The solutes may become very heterogeneous during the evaporation process because of different solubilities. Liquid constituents, such as Tween® 20, will return to a highly concentrated state and probably are not evenly distributed across the fibrous network of the pad. When sample is applied to the test strip, their rates of dissolution will depend on their inherent solubilities and the rate of mixing at the molecular interface. It should be expected that these constituents will not resolubilize at the same rates when the sample is applied to the test strip. Thus, more rapidly solubilizing constituents will be more concentrated near the liquid front, while those that solubilize more slowly will be delayed in their release into the liquid stream. The net effect of these phenomena is to generate a set of chemical gradients within the liquid stream as it moves through the pads and onto the membrane. There may be locations within the liquid stream where the concentration of the chemicals is not conducive to interaction between the antibodies and analyte.

Intended Use	Material	Code	Thickness (mm)
Sample/absorbent pad	Cellulose	C048	0.48
Sample/absorbent pad	Cellulose	C068	0.68
Sample/absorbent pad	Cellulose	C083	0.83
Sample/absorbent pad	Cellulose	C248	2.48
Conjugate pad	Glass fiber	G028	0.28
Conjugate pad	Glass fiber	G041	0.41

Table 3.
SureWick Pad Materials

Sample Pad Selection and Specifications

Selecting a Sample Pad

The sample pad (Figure 10) can be used to perform multiple tasks, foremost of which is to promote the even and controlled distribution of the sample onto the conjugate pad. It may also control the rate at which liquid enters the conjugate pad, preventing flooding of the device. When impregnated with components such as proteins, detergents, viscosity enhancers, and buffer salts, the sample pad can also be used to:

1. Increase sample viscosity (modulate flow properties).
2. Enhance the ability of the sample to solubilize the detector reagent.
3. Prevent the conjugate and analyte from binding non-specifically to any of the downstream materials.
4. Modify the chemical nature of the sample so that it is compatible with immunocomplex formation at the test line.
5. Promote even flow of the sample along the membrane.

The presence of added protein (such as albumin) and detergents and surfactants (such as SDS or Tween® 20 at a very low concentration) may promote resolubilization of the conjugate, reduce nonspecific binding of the conjugate, and possibly minimize adsorption of the analyte to the membrane.

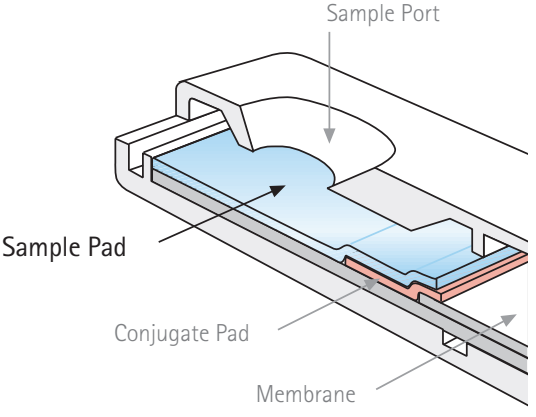


Figure 10.
Sample Pad

By adding blocking agents to the sample pad, it may be possible to eliminate blocking of the membrane. This approach may be much easier and considerably more cost-effective than attempting to block the membrane directly. Unless the antibody (or antigen) is covalently attached to the detector particle, it is not advisable to dry the detector reagent into the conjugate pad in the presence of blocking proteins or detergents. Exogenous proteins, especially in the presence of detergents, can displace the antibody or the antigen from the detector particle during prolonged storage. Thus, the sample pad may be the only place in the test device other than the membrane where blocking and resolubilization agents can be added safely.

Some tests require samples that exhibit wide variation in chemical composition. Human urine, for instance, can have a pH between 5 and 10. Differences in pH and ionic strength may shift the specificity and sensitivity of capture and detector reagents and promote varying degrees of non-specific binding of detector reagents due to changes in charge densities. Adding a relatively high concentration of buffer salts to the sample pad (for example, by pre-treating with 1.0 M borate buffer, pH 9.5) can minimize variation by controlling the pH and ionic strength of the solution that emerges from the sample pad.

There are two types of materials that are commonly used as sample pads: **cellulose fiber filters** and **woven meshes**. Woven meshes, sometimes called screens, normally work very well to distribute the sample volume evenly over the conjugate pad. They also typically have good tensile strength and handle well, even when wet. Meshes have very low bed volumes, meaning that they retain very little sample volume, normally 1 – 2 µL/cm^2. On the other hand, it is impractical to treat them with the intention of loading them with enough solutes to modify the protein content, pH, ionic strength or viscosity of the test sample. Meshes can also be expensive relative to other porous media and difficult to process through strip cutting machinery.

Cellulose filters have properties that are nearly the opposite of woven meshes. They are thick (> 250 µm), weak, and relatively inexpensive. Cellulose filters also have large bed volumes (> 25 µL/cm^2). Paper can be very difficult to handle, especially when wet. Cellulosic filters are the most commonly used materials to make the sample pad because they can be loaded with a wide array of blocking agents, detector reagent release agents, pH and ionic strength modifiers, and viscosity enhancers. When using cellulose filters, care must be taken to ensure sufficient and consistent contact with the underlying conjugate pad material. Failure to achieve good contact and adequate compression can lead to interrupted or inconsistent transfer of fluid into the conjugate pad.

For many urine-based assays, especially pregnancy and ovulation tests, porous plastic wicks protrude from the end of the cassette. Their primary function is to collect liquid from the urine stream so that it can be transferred to the test strip within the cassette. The dimensions of the wick are tailored to the design of the test strip cassette and the requirement to absorb enough liquid to run the test. The wick may or may not be chemically treated, depending on the chemistries of the other materials within the cassette. It is conceivable that the plastic wick can serve the function of a sample pad.

Specifying the Sample Pad

Specification of the sample pad depends greatly on its intended purpose in the test device. If the sample pad is being used primarily to modify the sample, the following attributes should be specified:

Thickness (Mean and Variability)

Mean thickness may be given as microns, millimeters, or thousandths of an inch (mils). The range of variability is also critical since this will affect the amount of bed volume and the consistency of compression in a housing. For strips placed in housings, the sample is typically applied to a port that exposes a small region of the sample pad. If the pad material is too thick, the fibers may be compressed so that absorption of liquid into the pad is greatly reduced or prevented. If the pad material is too thin, there may be little or no contact with the housing. This allows the sample to enter the housing unobstructed, flooding the interior, and significantly altering the flow dynamics of the test strip.

Basis Weight (Mean and Variability)

The basis weight is the mass of fibers per unit area. In the paper and non-woven industries, it is most often expressed as g/m^2. This value is of little relevance for lateral flow tests. Using the basis weight, the thickness of the material, and the density of the polymer, the bed volume and porosity can be calculated. Bed volume is directly proportional to thickness at constant porosity. Similarly, bed volume is directly proportional to porosity at constant thickness. Thus, variability in basis weight can be equated to variability in bed volume. Bed volume is actually the critical performance parameter, but it is rarely provided.

Tensile Strength

Tensile strength for sample pad materials is important for the same reason as for membranes. Some materials are as weak as unbacked membranes. Since they may be slit to widths of 1 cm or less, web handling can be very difficult in a continuous processing operation.

Extractables

Materials used to make sample pads contain binders to hold the fibers together. In addition, some of the fibers may break or not be interconnected with the pad's macrostructure. Consequently, a considerable percentage of the pad's components may be dislodged during various processing steps. When a test strip is run, this can lead to plugging and poor fluid transfer as the sample wets out the pad and moves downstream.

Dimensions (Length, Width)

The dimensions and tolerances should be completely defined.

Packaging, Labeling, and Certification

The considerations pertaining to membranes also apply to sample pad materials.

Particle Retention Rating

In some applications, the sample pad is used as a filter to remove particles from the sample before the liquid enters the conjugate pad. Thus, it is important to know the particle retention rating. Since these materials are depth filters, they do not exhibit 100% retention capability. Because of the way that papers and non-woven materials are manufactured, changing the particle retention rating often involves significant changes to the thickness and basis weight.

Conjugate Pad Selection and Specifications

The conjugate pad (Figure 11) can perform multiple tasks, the most important of which is uniform transfer of the detector reagent and test sample onto the membrane. When sample flows into the conjugate pad, the detector reagent solubilizes, lifts off the pad material, and moves with the sample front into the membrane. The ideal conjugate pad material has the following attributes:

1. **Low non-specific binding.** If the detector reagent or analyte binds to the conjugate pad, it will be unavailable to form the immunocomplex at the test line, thereby reducing signal intensity and sensitivity.
2. **Consistent flow characteristics.** Inconsistent flow properties can cause serious performance problems. If the material does not deposit the sample uniformly onto the membrane, the detector reagent may be channeled onto the membrane, appearing as streaks as the sample migrates along the membrane. Consequently, there will be uneven signal development at the test and control lines.
3. **Consistent bed volume.** When loaded into the conjugate pad by dipping, the amount of detector reagent in each test strip depends on the bed volume of the material. If the bed volume varies significantly, variable signal intensities may be observed even though all other components of the strip are constant.
4. **Low extractables.** In addition to chemical extractables, the material should be free of particles that can clog the membrane at the conjugate pad/membrane interface.
5. **Good web handling characteristics and consistent compressibility.** These attributes are important for incorporation into test strip manufacture and for consistent liquid transfer onto the membrane.

The Role of the Conjugate Pad in Assay Sensitivity

An important function of the conjugate pad is to deliver the detector particles onto the membrane in a consistent volume of sample on every test strip. Ultimately, the sample volume required to release the detector particle into the sample stream determines how much analyte can be measured. Only the analyte contained in the volume of sample that migrates ahead of and with the detector particles can contribute to the signal. The volume of sample that enters the conjugate pad and membrane after the detector particles have been completely released does not contribute to signal, although it does serve to reduce assay background (Figure 12). Analyte that passes over the capture reagent line after all of the detector particles have migrated farther downstream may bind at the capture reagent line but will lack additional detector particles to complete the immunocomplex. The sample volume actually analyzed in the test strip equals the amount of sample required to solubilize the detector particles, not the total amount absorbed by the device.

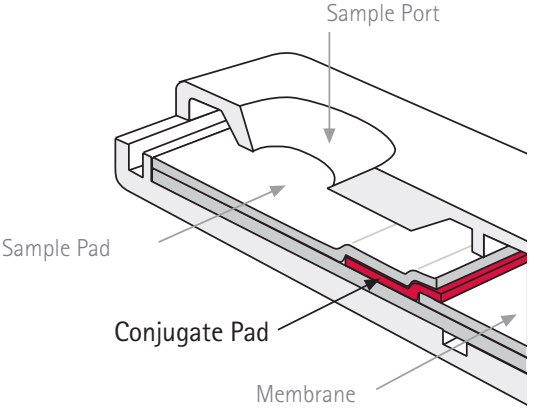


Figure 11.
Conjugate pad

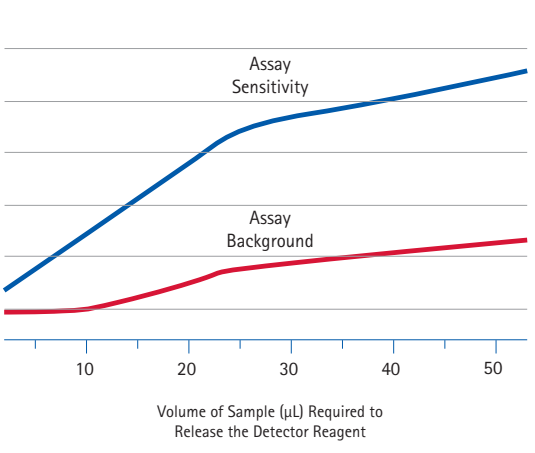


Figure 12.
Effect of inconsistent flow properties on assay sensitivity and background

The porous materials commonly used for conjugate pads are non-woven filters, which are manufactured by compressing fibers of cellulose, glass, or plastic (such as polyester, polypropylene, or polyethylene) into thin mats. They are specified by fiber size, thickness, basis weight, extractables, and air flow rate. In most cases, they cost considerably less than membranes. Materials commonly used to make conjugate pads include glass fiber filters, cellulose filters, and surface-treated (hydrophilic) polyester or polypropylene filters. The key properties for each are summarized in Table 4.

Specifying the Conjugate Pad

The conjugate pad is as critical as the membrane in controlling the performance of lateral flow tests. As such, it is very important to define key material specifications:

Thickness (Mean and Variability)

Thickness may be given as microns, millimeters, or thousandths of an inch (mils). The range of variability is also critical since this will affect the bed volume and the consistency of compression in a housing.

Basis Weight (Mean and Variability)

The basis weight is the mass of fibers per unit area. In the paper and non-woven industries, it is most often expressed as g/m^2. This value is of little relevance for lateral flow tests. Using the thickness of the material and the density of the polymer, the bed volume and porosity can be calculated. Bed volume is directly proportional to thickness at constant porosity. Similarly, bed volume is directly proportional to porosity at constant thickness. Thus, variability in basis weight can be equated to variability in bed volume. Bed volume is actually the critical performance parameter, but it is rarely provided.

Tensile Strength

Tensile strength for conjugate pad materials is important for the same reason as for membranes. Some materials are as weak as unbacked membranes. Since they may be slit to widths < 1 cm, web handling can be very difficult in a continuous processing operation.

Extractables

Materials used to make conjugate pads may contain binders to hold the fibers together. In addition, some of the fibers may break or not be interconnected with the pad's macrostructure. Consequently, a considerable percentage of the pad's components may be dislodged during various processing steps. When a test strip is run, this can lead to plugging and poor fluid transfer as the sample wets out the pad and moves downstream. Extraneous glass fibers can also present a health hazard in automated manufacturing systems.

Dimensions (Length, Width)

The dimensions and tolerances should be completely defined.

Packaging, Labeling, and Certification

The considerations pertaining to membranes also apply to conjugate pad materials.

Non-woven Material	Brief Description	Key Benefits	Key Liabilities
Glass fibers	100 – 500 µm thick, may contain binders to hold fibers together	Good hold-up volumes, low nonspecific binding	Poor tensile properties, difficult to slit and web handle
Cellulose filters	300 – 1000 µm thick, compact fibers of consistent density	Very low nonspecific binding, normally very uniform	High hold-up volumes (> 50 µL/cm^2), can be very weak when wet
Surface-modified polyester	100 – 300 µm thick, hydrophilic polyester filters	Low nonspecific binding, excellent tensile strength and web handling	Low, and somewhat variable, hold-up volumes (< 15 µL/cm^2)

Table 4.
Properties of conjugate pad materials

Blood Filter Matrices

For analytes found in serum, a highly desirable performance attribute for a lateral flow test strip would be its ability to accommodate whole blood as the sample. Porous media exist that are capable of separating cells from serum or plasma so that enough volume of sample is generated to run a lateral flow test. Efficient separation of blood cells from serum is extremely difficult. Blood contains 35% to 45% solids, mostly as red blood cells, meaning that a 200 µL aliquot of blood would be expected to yield at most 130 µL if the separation was 100% efficient. In a lateral flow test, the filter material must be capable of functioning over the full range of blood variability, yielding enough serum to fill the bed volume of the entire test strip reproducibly and with the same flow kinetics.

While several products have been commercialized as blood filters, they have not been widely accepted as components of lateral flow test strips. There are several patents describing materials and chemistries that can be used as blood separation filters. If the use of a blood separation filter is being considered, test strip developers may need to consider licensing one or more of these patents prior to commercialization of final product. EMD Millipore does not provide legal counsel, nor is it in the position of being able to assign any rights for any of the patents that have been issued. In the interest of providing useful information to users of lateral flow membranes, a list of pertinent patents can be found in the Appendix.

Absorbent Pad Selection and Specifications

Selecting an Absorbent Pad

Absorbent pads, when used, are placed at the distal end of the test strip (Figure 13). The primary function of the absorbent pad is to increase the total volume of sample that enters the test strip. This increased volume can be used to wash unbound detector particles away from the test and control lines, thereby lowering the background and enhancing assay sensitivity. Since the volume of sample that ultimately contributes to signal is controlled by the volume required to solubilize the detector particles, and not by the total volume of sample that enters the device, the addition of the absorbent pad may not have a dramatic impact on overall assay sensitivity. If the strip design does not include an absorbent pad, the volume of sample analyzed in the strip is determined solely by the bed volume of the membrane.

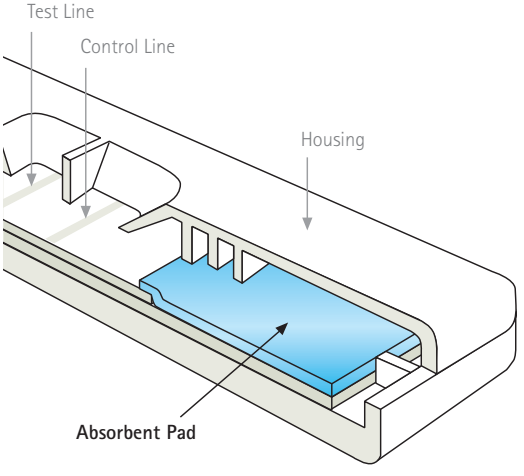


Figure 13.
Absorbent pad

There are two major considerations associated with the use of absorbent pads. First, a suitable material must be identified, specified, purchased, and integrated into the manufacturing process. Ultimately, this leads to a higher cost for the finished product. Second, an absorbent pad makes it difficult to incorporate an end-of-assay indicator in the test device. The flow of liquid through the absorbent pad is not necessarily laminar, and the pad may fill with liquid unpredictably.

Most absorbent pads are made from cellulose filters. The material should be selected on the basis of thickness, compressibility, manufacturability, and, most of all, uniformity of bed volume. Once an absorbent material has been chosen, optimizing the overall volume absorbed by the test strip is best managed by changing the dimensions (usually the length) of the absorbent pad.

Specifying the Absorbent Pad

All of the specifications for the sample pad apply to the absorbent pad, with the exception of extractables.

Adhesive Card Selection and Specifications

For ease of handling during manufacturing, the porous materials comprising the test strip are usually mounted on an adhesive card. The adhesive card consists of three components: the plastic backing, the adhesive, and the release liner. Dimensions (length, width, thickness) and tolerances of the plastic backing must be specified. If the finished strip will be placed in a housing, the thickness of the plastic backing must be consistent so that the porous materials are not subjected to variable degrees of compression.

The adhesive layer is in direct contact with the materials and chemistries critical to the functioning of the test strip. Key parameters to specify include thickness, extractables, volatile components, pressure sensitivity, temperature sensitivity, and shelf life. If the reagents are striped onto the membrane *after* it is mounted on the card, thickness variations for the plastic backing, adhesive layer, and membrane must be considered together. The striping mechanism should yield a uniform line appearance regardless of variations in aggregate thickness.

For the release liner, the placement and tolerances on score marks must be specified. Contact of the membrane with the release liner should be avoided. To promote release from the adhesive, many liners are coated with silicone-based compounds. These compounds can transfer to the membrane, rendering the top surface hydrophobic. This slight change in the surface chemistry can dramatically alter the consistency of liquid absorption during striping of the capture reagents.

Housing Selection and Specifications

Although not required, many lateral flow strips are placed into plastic housings. The housing prevents the user from applying the sample anywhere except the sample pad. In over-the-counter products for urine analysis, the housing also serves to protect the strip from inadvertent splash onto the membrane. External labeling on the housing can also be used to indicate the position of test and control lines and provide other information.

Housings can be obtained as off-the-shelf cassettes or custom-designed to fit around the strip. Deciding between these options requires balancing unit cost, design costs, size compatibility with the strip, and requirements for external labeling. Ultimately, the dimensions and tolerances on the internal features need to be matched to the dimensions and tolerances of the materials that comprise the test strip. Internal pins and bars are used to hold the strip in place relative to the sample port and viewing window. They hold the materials in intimate contact with one another while the test strip is running. If these features are too large, the pads and membrane may be compressed to the point that sample flow is blocked. The plastic used for the housing must also be biocompatible.

Manufacturing Schemes

General Considerations

A successful manufacturing operation requires the integration of materials, reagents, treatment processes, and assembly operations. The overall process can be entirely manual, completely automated, or a combination of manual and automated steps. Manual steps require the processing of materials in a batch mode, while automation allows for processing in a continuous mode. The decision to use a batch process or a continuous process relates to the amount of product to be manufactured and the funds available to staff and equip the manufacturing facility. Product of excellent quality can be manufactured by either method. Minimally, instrumentation is required for precise application of capture reagents and cutting of individual strips. The equipment used for batch processing is much less expensive than reel-to-reel systems commonly used for continuous processes. Not surprisingly, the labor cost for a fully developed continuous system is markedly lower than for product made by a batch process. Regardless of the manufacturing process chosen, the lot sizes of the materials and reagents should be matched during the planning process so that there is minimal waste.

The type of process chosen affects the dimensions of the materials. Batch processing usually calls for strips or sheets. The length and width requirements are well defined, and tolerances are small. Rolls can also be used but usually have to be cut into strips at some point during manufacture. For striping, the membrane must be perfectly flat and not overly rigid; some plastic backings may be unacceptable.

Continuous processes usually require rolls of each material. The width of the roll is normally well defined with narrow tolerances (e.g., 2.5 cm ±0.5 mm) based on the design of the processing equipment. The roll length, which typically ranges from 50 to 150 meters, depends on many factors, including lot sizes of other components, production rates, final product lot size, and manufacturing capacity. Tolerance on length is typically ±5%. Since the roll must be compatible with the processing equipment, additional requirements include edge alignment (maximum displacement of one wrap relative to another), edge quality (frequency of chips, dents, or nicks on the sides of the material), telescoping (displacement relative to the core), and packaging (packaging materials, core dimensions, side protection, labeling, interleaf, etc.). Tensile strength is a critical specification for rolls of unbacked membranes, especially those slit to widths < 5 cm. Unbacked membranes break easily in reel-to-reel manufacturing processes unless tension control and membrane alignment are precisely maintained. The use of backed membranes is recommended for a continuous process.

An important manufacturing consideration is the potential effect of processes and processing equipment on the physical structures of the materials. Specific details are outlined below. A laboratory researcher will probably be very meticulous in the assembly of test strips because of in-depth knowledge of the mechanisms underlying test strip performance. To achieve the same level of consistency in a manual assembly operation is likely to require a significant level of continuous training of the manufacturing and QC personnel and a high level of in-process inspection so as to minimize waste and maximize yield. Ultimately, the materials must not be damaged during manufacture or handled in ways that make their performance unpredictable.

Optimizing the Capture Reagent Buffer

The buffers used for dilution of the capture reagents have to be optimized so that protein adsorption is uncompromised, reagent reactivity is retained, and flow properties of the membrane are unaltered. Ultimately, the capture reagent must meet the specifications for sensitivity, specificity, and stability. Depending on the nature of the reagent, there may be considerable latitude in the buffer formulation. Others, like certain antibodies and synthetic constructs, may require a specific formulation to maintain structural stability and reactivity. Solution parameters that should be optimized are as follows.

Buffer Selection

Capture reagent solutions should always be buffered to achieve consistency of manufacturing and test strip performance. Minimally, the pH of each batch of reagent should be the same, even if it is not the optimal pH. Many buffers can be used to control the pH of capture reagent solutions including phosphate, borate, TRIS, and carbonate. For reagent application, some buffers are better than others; some relatively uncommon ones might be even better. It is important to remember that the capture reagent is dried onto the membrane in the presence of the buffer solutes. Problems can arise from chemical interactions that occur during evaporation, when the concentration of these solutes becomes transiently very high. For example, if the reagent solution is buffered with a primary amine such as TRIS or glycine, salt bridging can occur between acidic amino acid residues (glutamic acid and aspartic acid) in the capture reagent and the $-NH_3^+$ group of the buffer molecule, reducing the capture reagent's ability to bind analyte. Sodium is a better cation than potassium for the same reason. For capture reagents that are amenable to a pH range of 4 to 6, ammonium acetate can be used. It leaves essentially no residue upon evaporation since both acetic acid and ammonia are volatile.

Choosing pH

As the solubility of the capture reagent is reduced, the tendency of the antibody to partition onto the membrane increases. Protein solubility is influenced by pH. Solubility is minimal when the protein has no net charge, i.e., at the isoelectric point, **pI**. Thus, to minimize protein solubility and maintain a stable solution, adjust the pH to ±1 unit of the pI of the capture reagent. Most antibodies have isoelectric points between pH 5.5 and 7.5. Using a buffer at pH 7.0 – 7.5 is probably close to optimal. When the pI of the capture reagent is unknown, the optimal pH will have to be chosen empirically.

Ionic Strength

The ionic strength of the buffer should be reduced as much as possible. Ions in solution can interfere with electrostatic interactions between the membrane and the capture reagents. In addition, physiological concentrations of buffer salts and sodium chloride promote the solubility of most proteins and reduce the hydrophobic attraction of the nitrocellulose membrane. Finally, as the molarity of the capture reagent solution increases, the amount of solid residue left on the membrane after evaporation also increases. Solid residue in the pores can delay wetting of the membrane and alter its flow properties when the test is run. Thus, the molarity of the buffer should be lowered to the minimum required to maintain a stable pH (usually < 10 mM). Sodium chloride should be eliminated unless absolutely required for protein solubility or stability. The use of buffered saline solutions as diluents is generally *not* recommended.

Use of Methanol, Ethanol, or Isopropanol

The addition of a small amount of alcohol (1% to 10% v/v methanol, ethanol or isopropanol) to the reagent application solution can be extremely beneficial for three distinct

reasons. First, the alcohol can dramatically improve the consistency of reagent application by lowering solution viscosity, by lowering solution surface tension (both of which promote entry of the solution into the membrane), and by decreasing static repulsion. Second, the addition of a small amount of alcohol decreases protein solubility without causing denaturation or precipitation. It also promotes the adsorption of the protein onto the nitrocellulose membrane. Third, the presence of even a low concentration of alcohol enhances drying and protein fixation. The benefits of adding alcohol should be determined empirically.

Use of Detergents

Occasionally, the addition of a capture reagent solution displaces surfactant present in the membrane, causing the reagent line to wet more slowly than adjacent areas of untreated membrane. When this occurs, the flow front becomes deformed. In extreme cases, the flow front wraps around the capture reagent line, entrapping air (especially when the membrane is sandwiched between two layers of nonporous plastic) and preventing flow over the line (Figure 14). To prevent this, it is sometimes effective to add a small amount of detergent (e.g., 0.05% SDS or 0.005% Triton™ X-100) to promote rewetting of the line.

Applying Reagents to Membranes

Effects of Humidity and Electrostatic Forces

Humidity affects the handling of nitrocellulose membranes in manufacturing processes. Under conditions of low humidity, nitrocellulose membranes develop a significant static charge. This causes the membrane to be attracted to or repelled by different surfaces. Unbacked membranes can be very difficult to handle under these circumstances. Backed membranes are usually easier to handle because the weight of the backing overcomes the static forces. With either type of membrane, dust and dirt particles are attracted onto the membrane and are very difficult to remove without damaging the membrane surface.

A more serious problem with static charge is interference with reagent application. Water droplets are naturally electronegative. If the droplets are repelled by static charge, the width of the reagent line width may vary dramatically. In extreme conditions, the line may be broken instead of continuous. This problem is more significant for air-jetting systems because the liquid stream is converted to an aerosol. It is typically less of a problem for positive displacement dispensers because the stream is dispensed continuously.

In general it is better to apply capture reagents under conditions of excess humidity than under conditions of insufficient humidity. For most manufacturers this generally translates into the need to humidify the reagent application area during the dry months. Optimal results are obtained when the room is maintained at a relative humidity of ~50% at standard room temperature (18 – 22 °C, 65 – 72 °F). Depending on external atmospheric conditions and the efficiency of the air handling system, indoor relative humidity can be < 20%. Reel-to-reel systems can impart a static charge solely from friction as the membrane passes over rollers and other surfaces. In this situation, it may be necessary to install an ion gun in-line just before the membrane passes under the dispenser tip. The ions from the ion gun provide temporary dissipation of the static charge.

Optimizing Reagent Application

Regardless of the manufacturing process used, the striping protocol for reagent application must be optimized. Variables to be considered include:

1. For unbacked membranes, the side to which reagents will be applied
2. The side of the membrane from which results will be assessed
3. The reagent dispensing rate (µL/cm)
4. The concentration of capture reagent to be dispensed
5. The capture reagent diluent (see previous section)
6. Drying

Commercially available assays exist that are formatted so that results are displayed on the side of the membrane to which capture reagents were applied or on the opposite side. If the assay is read from the opposite side, the reagent solution must penetrate through the membrane, carrying with it sufficient capture reagent to produce a consistent line at the desired sensitivity. Dispensing rates and reagent concentrations may require adjustment.

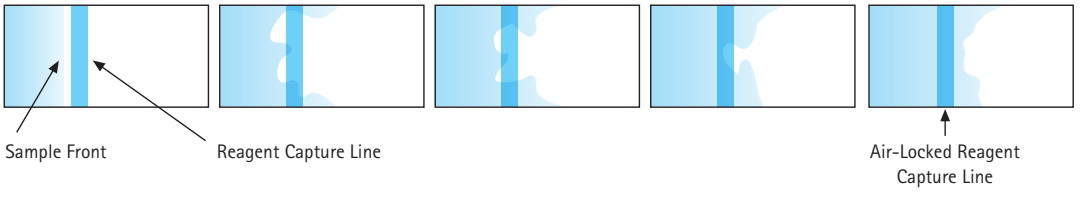


Figure 14.
Flow front deformation due to slow wetting of capture reagent line

Reagent application buffers should be determined within the context of the interactions that take place between the membrane and proteins and the chemical environment necessary to optimize these interactions. The key aspects have been discussed above and are briefly summarized here:

1. Reduce the molarity of the buffer as much as possible, preferably < 10 mM.
2. Eliminate sodium chloride and other salts from the buffer.
3. Eliminate surfactants and detergents such as Tween® 20 and Triton™ X-100.
4. Eliminate other additives.
5. Fix the capture reagent concentration at a level that produces the desired line width on the final assay, while simultaneously achieving the specified sensitivity and specificity.

If salts and detergents are essential for stability of the capture reagent either in solution or in dry form, they should be added at the minimum concentration necessary. Ionic detergents, such as SDS, can be used safely at concentrations < 0.1% w/v to reduce nonspecific protein interactions and promote rewetting of the capture reagent lines in the final assay. To aid in uniform dispensing of the reagent solution, the buffer can be supplemented with alcohol.

When the capture reagent is dispensed at concentrations < 100 µg/mL, it is unlikely to saturate the surface of the nitrocellulose, resulting in individual molecules being bound to the membrane with adjacent areas of open nitrocellulose. In some instances, most notably for antibodies, a slow loss in reactivity is observed. This is presumably due to denaturation over time as molecules rearrange on the nitrocellulose surface. The addition of non-specific proteins to the reagent buffer can be used to stabilize the capture reagent. Bringing the overall protein concentration to 1 mg/mL is normally sufficient. The selection of the bulking protein is important. It should not result in non-specific capture of detector particles nor should it interfere with capture reagent recognition. Bovine serum albumin (BSA) and species-matched immunoglobulins are generally good choices.

The optimal rate for dispensing the reagent solution, typically expressed as µL/cm, is a function of several factors. First, a sufficient mass of capture reagent has to be dispensed to achieve the desired specificity and sensitivity. This is determined empirically during strip development. Second, the width of the capture reagent line is controlled for the most part by the volume of liquid applied. When the volume is reduced in an attempt to narrow the line, the concentration of the capture reagent must be increased so that the mass applied remains constant. Failure to do this may obscure any performance benefits or losses. Third, the volume of liquid dispensed by the pump has to be matched to the speed at which the membrane passes under the dispenser tips so that the same volume of solution is delivered per unit distance. These parameters can be tested in various combinations to achieve the desired line quality.

The last consideration is the type of mechanism to be used for striping and the underlying engineering capabilities and limitations. In positive displacement systems, a liquid stream is dispensed directly onto the membrane. This type of delivery is performed with non-contact or contact tips. For non-contact dispensing, a tip is set a fixed distance above the membrane, normally on the order of 50 µm. If the variation in membrane thickness is too great, the membrane may come into contact with the tip, resulting in physical damage to the membrane. Less significant variations can also lead to variation in the dynamics of fluid penetration into the membrane, depending on the nature of the striping solution interaction with the membrane. With contact tips, a short segment of flexible tubing is dragged across the surface of the membrane simultaneously with the dispensing of the liquid. If the tip is applied to the membrane with too much pressure, a groove is embossed into the nitrocellulose. If the groove is too deep, capillary flow can be adversely affected. Variable membrane thickness can also lead to varying depths of the groove. This problem can be assessed visually using a stereo microscope.

In air-jetting systems, pressurized air is injected into the liquid stream prior to dispensing. The liquid contacts the nitrocellulose in the form of an aerosol. Because the droplets are extremely small, they are more sensitive to static repulsion. The amount of air used must be considered. Air pressure that is not dissipated through the membrane will be dissipated laterally, carrying the aerosol with it. Thickness variation in the membrane can also cause problems by changing the area covered by the aerosol. As the distance between the tip and the membrane increases, the dispersion of the aerosol increases.

Fixing the Capture Reagents

It is critical to dry the membrane completely to fix the capture reagents to the nitrocellulose. Backed membranes require longer drying times than unbacked membranes. Drying efficacy is a function of temperature, humidity, airflow, and time. Drying in a vacuum chamber is ideal but seldom practical for manufacturing. Although there may be considerable variability in results, conditions required to achieve complete drying are summarized in Table 5. Failure to dry the membrane completely may lead to the loss of capture reagent from the capture lines, especially if the membrane is blocked or washed before final assembly. Proteins used to block the membrane may displace capture reagents that are inadequately fixed.

In continuous systems, the membrane is typically dried by passing it through one or more drying towers. Air temperature and air flow rate in the drying tower are balanced against the line speed and capture reagent application rate so that the membrane is fully dry before final wind-up. This can be achieved in just a few minutes.

Once the membrane has been dried, it should be stored at 4 to 20 °C at < 15% relative humidity to prevent rehydration of the capture reagent, whether prepared in a batch or continuous process.

Blocking the Membrane

A common question in the design of a lateral flow test is whether or not it is necessary to block the membrane after applying the capture reagents. There are numerous, commercially available tests where the membrane has not been blocked. Extraneous proteins present in the sample or blocking agents added to the sample pad are frequently sufficient to prevent the detector reagent and analyte from non-specifically adsorbing to the nitrocellulose. In other instances, blocking is used to produce a chemically uniform surface along the entire length of the membrane. If blocking is required, numerous blocking agents may prove compatible with the test: 0.1–0.5% w/v gelatin, 1 – 2% w/v casein, 1 – 2% w/v BSA, 1 – 2% w/v IgG, 0.5 – 1% w/v PVP (8 – 10 kDa), and 0.1 – 1% w/v PVA (8 – 10 kDa). After selecting one or two candidate blocking agents, it is best to determine the optimal concentration empirically. In choosing a particular blocking agent, it is important to recognize that many of these substances come in various grades of purity and overall composition.

Details of the blocking protocol depend on the overall manufacturing process. In a manual operation, membrane can be processed in batches by immersing strips in the blocking solution after the capture reagents have been applied and dried in place. The immersion process must be slow enough to allow air to be displaced from the pores of the membrane. For backed membranes, the blocking solution must be allowed to wick in from the edges. Upon complete wetting, the strips can be submerged. There needs to be sufficient blocking solution to completely immerse the strips and sufficient mass of the blocking agent to coat the exposed polymer. Repeated use of a blocking solution is not recommended due to the depletion of the blocking agent. Typically, it is only necessary to leave the membrane in contact with the blocking solution for 1 – 15 minutes.

After the membrane has been blocked, it is critical to remove excess blocking agent by washing with a weak buffer solution (e.g., 5 mM Na_2HPO_4, pH 7.5). Uneven flow rates and uneven flow fronts are frequently caused because this step is overlooked. Excess blocking agent left on the membrane can dry down as crystals that physically clog the pores. To promote even rewetting of the blocked membrane, a low concentration of detergent (0.01% w/v SDS) may be added to the wash buffer. In a batch process, sheets or strips are immersed in wash buffer for 5 to 15 minutes. Relative to the time required for blocking, more time is necessary for washing because the excess blocking agent must diffuse out of the membrane and into the wash buffer.

Temperature	Humidity	Drying Time	Comments
15 – 25 °C	Desiccated	>16 hours*	May require much longer times
35 – 40 °C	Desiccated	1 – 2 hours*	Safe for Abs, reliable conditions
45 – 55 °C	Desiccated	<0.5 hours*	Most Abs are OK, fast drying; prolonged exposure can damage the membrane

Table 5.
Conditions required to achieve complete drying

* If heated air is blown over the membrane, the time required to achieve complete dryness may be considerably shorter.

In continuous manufacturing, the blocking step can be integrated with striping of the capture reagents as part of a single process. A bath containing the blocking solution is located in sequence after the striping station. There are several considerations when using this approach:

1. Is a drying tower required between the striping station and blocking bath to fix the capture reagents?
2. What are the dimensions of the blocking bath?
3. Is the line speed sufficiently slow to allow for complete wetting of the membrane as it enters the blocking bath?
4. What is the residence time of the membrane in the blocking bath?
5. How will the blocking agent concentration need to be adjusted in consideration of the residence time?
6. Will a wash bath be required to remove excess blocking agent from the membrane?
7. What is the rate of blocking solution replenishment?
8. What air temperature and air flow rate will be used in the final drying tower?

The composition of the blocking solution is typically optimized against the processing speed and mechanical systems so that a wash bath is unnecessary.

Regardless of how the membrane is blocked and dried, it should be stored at 4 to 20 °C at < 15% relative humidity to prevent rehydration.

Preparing Conjugate Pads

Buffer Composition

The buffer used for applying detector particles to the conjugate pad should be as simple as possible. At first consideration, this is counterintuitive, since the biological reagent attached to the detector particle must remain reactive after drying. In reality, however, compounds used to stabilize antibodies and other biological reagents in aqueous form are often incompatible with dry storage, either because they alter biological activity or damage the particles chemically and physically. Hence, salts and detergents should not be used in the application buffer. Although they are typically required for overall test functionality, they should be placed into the sample pad so that they are spatially separated from the conjugated particles. Colloidal gold particles are more sensitive to chemical and physical damage than latex microspheres.

The recommended buffer for colloidal gold particles is 2 mM borate at pH 7, supplemented with 1% to 10% sucrose or trehalose. The borate provides buffer control and also has a slight surfactant quality that aids in resolubilization of the particles. The sucrose (or trehalose) serves as a preservative and a resolubilization agent. When detector particles are dried in the presence of sugar, the sugar molecules form a layer around the particles that helps to stabilize the biological structures. When the sample enters the pad, the sugar molecules instantaneously dissolve, carrying the particles away from the surface and into the fluid stream. This buffer is also suitable for latex microspheres.

If the detector particles are stored in a buffer different from that to be used for application to the conjugate pad, the buffer should be exchanged so that components of the storage buffer are not carried over into the conjugate pad. The particles can be collected by centrifugation, the supernatant poured off, and the particles resuspended in the application buffer.

Loading the Conjugate Pad

The first consideration in loading detector particles into the pad material is the sequence of steps that is envisioned. The basic procedure will be dictated by the format of the supply, and several variations are possible (Table 6).

To treat the material, a flat container should be filled with enough detector particle solution to allow submersion. The size of the container and the depth of solution used should be considered in advance to allow for ease of handling without using a large excess of solution. The pad material should be dipped in the solution and held up to allow all excess to run back into the pan. The material should then be laid flat on a nonabsorbent surface for drying. In a reel-to-reel process, drying is normally done in-line. When processing strips, materials with weak tensile strength will require careful handling to prevent breakage and creases. Glass fiber and cellulose filters are typically much weaker wet than dry; processing is complicated by the fact that strips are typically < 1 cm wide. Synthetic fibers do not have this disadvantage. Also, sheets are not as susceptible to mechanical damage as strips. During dipping the materials should be handled with forceps and gloved hands.

Table 6.
Procedure for loading conjugate pads

Format	Strips	Sheets	Sheets	Rolls	Rolls	Rolls
Step 1	Dip	Dip	Cut into strips	Cut into strips	Continuous dip (reel-to-reel)	Continuous spray (reel-to-reel)
Step 2	Dry	Dry	Dip	Dip	Dry (in-line or on rolls)	Dry (in-line or on rolls
Step 3	–	Cut into strips	Dry	Dry	Cut into strips	Cut into strips

Drying

To maximize the performance of the detector particle, the treated pad needs to be dried as quickly and completely as possible. Specific options are listed below.

1. **Air drying.** Air drying in an uncontrolled room should only be used for investigative purposes during experimentation at the R&D level. It should not be considered as a routine process for manufacturing unless done in a dry room at a relative humidity < 15%. Air drying should not be considered complete as it does not remove water complexed with sugars and biomolecules. Complexed water can alter long term stability.
2. **37 °C for 2 hours.** Supplementing the drying process with heat helps to remove complexed water. This option works for many applications, and incubators are relatively inexpensive. Note that certain biomolecules may be heat labile and unable to survive the incubation period. Shorter incubation periods to preserve reactivity will have to be balanced against the completeness of drying.
3. **Freeze drying.** This process greatly speeds the rate of evaporation, but freezing may damage the particle structure. Also, the freeze-dryer must have sufficient capacity for the amount of material to be processed.
4. **Vacuum drying.** This process speeds the rate of evaporation without the deleterious effects of freezing. A vacuum pump and vacuum chamber are required.

The primary reason for evaporating the water as quickly as possible is to minimize the migration of the particles in the matrix. Since the particles are not adsorbed to the surface of the material, there are no chemical forces to hold the particles in place. Thus, if the liquid moves, the particles will also tend to move. The edge effect is a well-known phenomenon on sheets. This is caused by preferential evaporation at the edges of the sheet, which draws more particles from interior sections. When completely dry, the edges are noticeably more tinted than the center section. To compensate for this effect, the edges are cut from the sheet and not used for test strip assembly. Strips typically do not suffer from this artifact, although one edge may be noticeably darker than the other.

When developing quantitative assays, the techniques outlined above are often inadequate because they allow for too much variation in the amount of conjugate put into each test strip. To reduce this variation, direct spraying of conjugate particles onto the conjugate pad has been used as a manufacturing strategy. There are two advantages to this approach. First, the amount of conjugate applied can be standardized along the length of the strip and is less dependent upon the variation in bed volume of the pad material. Second, dispensing can be done in an automated fashion, thereby taking advantage of the improved efficiency of continuous processing of large rolls of material. Care has to be taken to ensure that the dispensing apparatus can accommodate hard particles of a fixed size. This often necessitates a different system from that used for capture reagent solutions. Conditions within drying towers typically will also need to be adjusted to evaporate a greater mass of water. A potential problem with spraying onto conjugate pads is heterogeneous spreading of the liquid in the pores of the glass fiber material resulting from its irregular pore structure. This can be compensated for by spraying multiple lines adjacent to one another so as to average out areas of broad and narrow spreading.

Storage

Once the detector particles have been dried into the pad material, it is important that the pad be stored at < 15% relative humidity. Temperature can range from 4 to 20 °C, keeping in mind that relative humidity changes with temperature. If the pad is exposed to higher humidity, water can complex with the sugar molecules, converting them to a syrup and delaying particle resolubilization.

Preparing Sample Pads

As discussed previously, the sample pad can be impregnated with a variety of chemical agents designed to make the full range of samples compatible with the functioning of the test strip. Unlike chemical considerations for the capture reagents and detector particles, there are far fewer constraints on what can be added to the sample pad. Fundamentally, enough chemistry must be loaded to make all samples behave similarly in the test. For example, human urine ranges in pH from 5 to 10. Buffer salts must be loaded into the pad so that samples over this range will all have the same pH when they transfer into the conjugate pad. The concentrations loaded into the pad will be determined by the bed volume of the material, the volume of sample analyzed in the test strip, and the variability of the samples.

The techniques that can be used to load the sample pad are essentially the same as those used to load the conjugate pad. The format of the pad material (strips, sheets, or rolls) and the processing technique (batch or continuous) determine the constraints on the process. One point to keep in mind is that cellulose filters, which are commonly used as sample pads, are very weak when wet. They will have to be handled carefully to prevent breakage, creases, and frayed margins. Once impregnated with the treatment solution, the sample pads should be dried and stored as described for conjugate pads.

Lamination of Pads and Membranes

Alignment

For the test strip to function, the pads and membrane have to overlap one another so that there is a continuous flow path for the sample (Figure 1). For the test strips to function **consistently**, the overlaps have to be consistent so that the flow dynamics are uniform on all of the strips manufactured. Test strips exist on the market where the pads and membranes are held in proper alignment by the housing alone. It is more common, however, for the pads and membranes to be adhered in proper alignment on an adhesive backing (Figure 15). These master cards are then slit into individual strips of a specific width for direct packaging or placement in a plastic housing.

Why is consistency of the overlaps so important? Fundamentally, this question relates to flow dynamics. The degree of overlap determines how the sample flows from one material to the next. Ideally, sample flow is laminar throughout the device. This is most critical for the sample pad, conjugate pad, and membrane because **the liquid takes the path of least resistance through the device.** If the overlaps are not configured properly, the pads may wet out yet contain areas where sample is not actually flowing. This may create significant problems in assay consistency because of slow and potentially a variable diffusion rate of detector particles into the liquid stream. The alignment can be configured so that the detector particles are washed out of the conjugate pad rapidly and completely or more slowly. The critical aspect is to establish and adhere to specifications and tolerances for the overlap of each material.

Also important in the lamination process is the degree of contact with the adhesive card. Normally this is not an issue for the membrane or absorbent pad, because the contact area is large. For the sample pad and conjugate pad, however, the contact area with the adhesive can be quite small. These pads are usually small to begin with, and a significant proportion of the area may be used for the overlap. If there is insufficient contact with the adhesive, the shearing force of the slitting process may cause these materials to delaminate from the card.

The ability to align the pads and membrane consistently is partly related to the adhesive card and the materials themselves. The adhesive card is typically scored so that a single release liner is removed before the lamination of a particular material. When this is done, the edges of the liners can serve as reference points for laying down the membrane and pads. Placement of the scores should be specified with tolerances established. Similarly, the variation in width of the pads and membrane must be minimal. In a manual assembly process, alignments should be checked after the addition of each material. This can be tedious since it will require measuring small distances, typically < 2 mm, at an accuracy consistent with the specifications and tolerances. Testing of 100% of the assembled cards is impractical. In a reel-to-reel system, in-line vision systems can monitor positioning on a continuous basis without any direct operator intervention. Defective regions can be marked automatically for removal from the production stream.

Lamination

The key aspect of the lamination process is to apply the materials to the adhesive card in the proper alignment

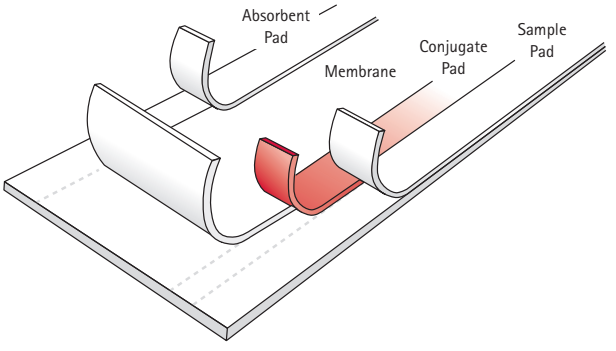


Figure 15.
Lamination on adhesive cards

without compressing their porous structures. Membranes are mechanically sensitive to overcompression as changes in the pore structure are irreversible. In extreme cases, flow will be completely blocked. The pad materials are not as sensitive, but they may be damaged by overcompression and not exhibit the same flow properties.

In a batch process, lamination can be performed using a device known as a clam-shell laminator. Materials are held in place on a movable arm, and the adhesive card is held in place on a movable platform. The arm is pivoted so that the material is brought into contact with the adhesive card. Pressure is applied to the arm, and the material adheres to the adhesive. Lamination can also be done entirely by hand. Here, the limiting factor tends to be the consistency with which individual operators align the materials on the adhesive card and apply pressure to the materials. Shifts of a millimeter or less and different degrees of compression may be imperceptible to the eye yet cause unacceptable variation in flow properties.

In a continuous process, engineering controls on the reel-to-reel system align the materials as they are processed through a gap in a nip roller. The gap, the pressure applied, temperature, and line speed are optimized to give adhesion without causing compression. Multiple lots of membrane and pad materials should be evaluated before finalizing the process window.

Lamination Defects

Lamination defects show up in several of ways. Overcompression of the sample pad, conjugate pad, or membrane is manifested as slow or no flow. Irregular flow fronts and streaking of detector particles on the membrane indicate inconsistent transfer of sample from the conjugate pad onto the membrane (Figure 16). This may be due to too little overlap or inconsistent contact between the conjugate pad and membrane. It is important to recognize that inconsistent flow arising in the sample pad and conjugate pad cannot be corrected by the membrane. If the absorbent pad is overcompressed during lamination or in a housing, flow may appear normal as the sample front passes the viewing ports. If it slows or stops when it reaches the absorbent pad, this may not be readily apparent to a user.

Slitting

Once the master cards have been assembled, they require slitting into individual strips before being placed into housings and packaged. Commercially available slitters employ a guillotine shear mechanism or a rotary cutting mechanism; both can be used successfully. The type of cutter used will be determined by availability of funds and output requirements.

There are two practical considerations for slitting. The first relates to the fragility of the materials used in test strips. The blades must be capable of cutting through the adhesive card, the strongest material, without damaging the membranes and pads, which are considerably weaker. If the engineering of the cutting mechanism is inappropriate for membranes and pads, they may delaminate from the adhesive or be damaged along the cut edges. These problems affect the uniformity of sample flow along the strip. Separation of the pads and membrane from the adhesive backing along the edge of the strip provides channels through which sample can flow unimpeded. This creates numerous problems with consistency of performance.

The second consideration relates to maintenance. Equipment manufacturers usually provide information on cleaning and general maintenance intervals. The equipment may require more frequent cleaning than prescribed due to accumulation of adhesives on the blades. If the build-up is great enough, the adhesive can stick to strip materials during slitting, causing delamination. Also, bits of nitrocellulose from the membrane and fibers from the pads may accumulate.

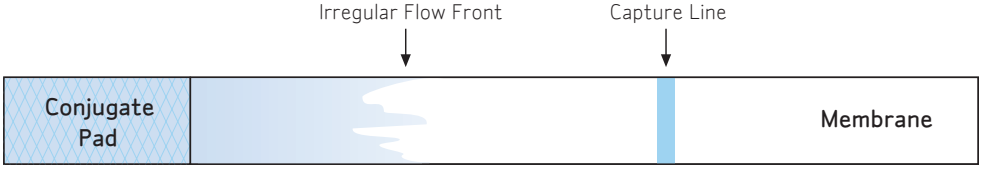


Figure 16.
Flow front anomalies due to lamination problems

Summary and Conclusions

Developing an immunochromatographic assay is complex because of the large number of critical components brought together to produce a functional test strip. Changing one material or reagent typically affects the performance of others. Ultimately, it becomes necessary to understand how each component contributes to the overall performance of the test strip. It is straightforward to vary capture reagent concentration, detector particle concentration, and the volume of sample being analyzed. It is fundamentally much more difficult to design experiments to evaluate the effects of membrane variation. This is what EMD Millipore had in mind when it developed the family of Hi-Flow™ Plus Membranes. Each membrane has a specific range of capillary flow times, represented graphically in Figure 17.

By testing a range of Hi-Flow™ Plus membranes, the effect of flow rate on assay performance can be determined. The data obtained in such an experimental plan might be summarized as shown in Figure 18.

Most assay performance attributes are affected by the flow rate of the membrane. The magnitude of the effects and the limits of acceptability are unique to the assay, reagents, and manufacturer. For example, lateral flow test strips that use HF075 will finish in the shortest time. They are likely to consume the most reagents and have reduced sensitivity. This may be acceptable for a pregnancy test where speed is critical and reagent costs are relatively low. In contrast, test strips developed using HF180 membranes require the longest time to complete. These assays will probably consume less reagents and achieve the maximum sensitivity allowed by those reagents. These circumstances may be the most desirable for infectious disease testing. The other membrane types produce assays that have different balances between speed, sensitivity, and cost.

The availability of this range of membranes allows the lateral flow test manufacturer to select the one that best fits the performance requirements established for the test under development.

Figure 17.

Length of time to flow to completion on 4 cm strips of Hi-Flow™ Plus Membranes

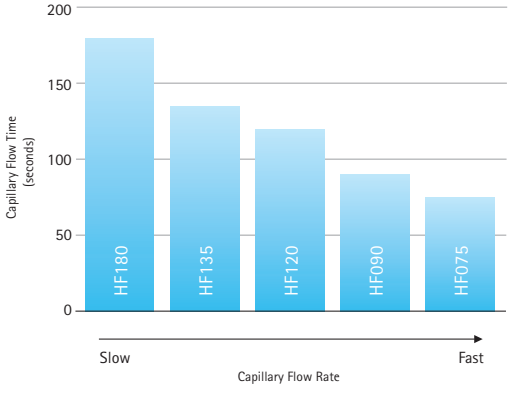
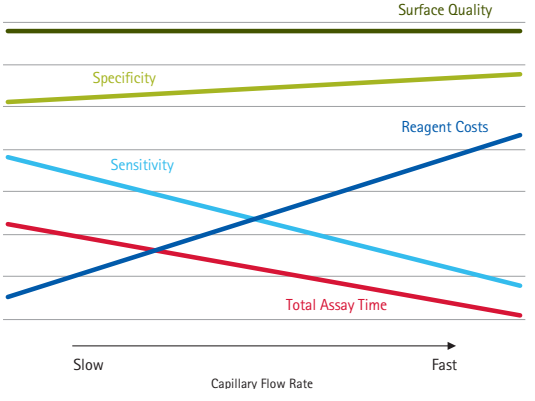


Figure 18.

Typical relationships between membrane flow rate and immunochromatographic assay performance



Appendix

Partial List of Patents Relevant to the Development and Commercialization of Immunochromatographic Assays

Patent Number	Assignee & First Inventor	Priority Date	Brief Description of the Invention/Application (Abbreviated Primary Claim)
(US) 4313734	Akzonn, Inc. JHW Leuvering	02 FEB 82	A method, test kit, and labeled component for the detection and/or determination of one or more analytes. The labeled component is comprised of colloidal metal (e.g., gold), >5 nm in diameter to which a detector reagent (antibody or antigen) is coated.
(US) 4376110	Hybritech GS Davis	08 MAR 83	Immunometric analysis for detection and determination of antigens that uses two monoclonal antibodies to form a sandwich ternary complex. This is a reagent patent that applies to all immunometric (sandwich-type) assays.
(US) 4435504	Syva Company R Zuk	06 MAR 84	Chromatographic immunoassay employing a specific pair member and a label conjugate which delineates a border whose distance from one end of the chromatography relates to the amount of analyte present.
(US) 4703017	Becton Dickinson RL Campbell	27 OCT 87	Solid phase assay for an analyte wherein binder is supported on a solid support (e.g., nitrocellulose membrane) and the tracer is comprised of ligand labeled with a colored particulate.
(US) 4855240	Becton Dickinson RW Rosenstein	08 AUG 89	Test device and assay for determining analyte wherein tracer and sample may be simultaneously applied to different absorbent material portions both in capillary flow communication with a solid phase (e.g., nitrocellulose membrane) with binder for analyte.
(US) 4954452	Abbott Laboratories DA Yost	04 SEP 90	A method of performing a diagnostic immunoassay utilizing colloidal non-metal (e.g., selenium, tellurium, sulfur, etc.) particles having conjugated thereto a binding component capable of specifically recognizing an analyte to be determined.
(US) 5028535	Biosite KF Buechler	02 JUL 91	Ligand assay with controllable thresholds for signal production by competitive reaction with labeled conjugate and receptor (e.g., antibody), both present in predetermined and controlled amounts.
(US) 5075078	Abbott Laboratories EW Osikowicz	24 DEC 91	Self-performing immunochromatographic device: Chromatographic test strip containing immobilized reagents that produces distinctly different read-outs (e.g., –, +) depending on the medical diagnosis.
(WO) 95/16207	SmithKline Diagnostics, Inc. HM Chandler	07 DEC 93	An assay device for detection and determination of an analyte in a test sample that uses a barrier containing an aperture to control the application and flow (including in the reverse direction) of reagents, including the sample.
(US) 5654162	Bio-Metric Systems PE Guire	05 AUG 97	A device capable of generating a signal indicative of the presence of an analyte, the device comprising a liquid permeable solid medium to which a reactant that can bind the analyte is immobilized and a detector reagent (labeled antibody) that can also bind to the analyte.
(EP) 0810436A1	Unilever PLC PJ Davis	03 DEC 97	An analytical test device incorporating a dry porous carrier to which a liquid sample suspected of containing an analyte can be applied indirectly, the device containing a mobile detector reagent and an immobilized capture reagent, and the means to control and detect analyte and detector reagent binding.

Partial List of Patents Related to Whole Blood Filtration

Patent Number	Assignee & First Inventor	Priority Date	Brief Description of the Invention/Application (Abbreviated Primary Claim)
(US) 5186843	Ahlstrom JS Baumgardner	16 FEB 93	Single layer, porous composite material useful as filtration media, especially for the separation of plasma or serum from blood.
(US) 5240862	X-Flor B.V. & Primecare B.V DM Koenhen	31 AUG 93	A process and a device for the separation of body fluid (e.g., plasma) for particulates (e.g., RBCs and WBCs) to be used for a chemical analysis (e.g., immunoassay) of the fluid components.
(EP) 586789	Boehringer Mannheim H Harttig	16 MAR 94	Use of a pre-treated, highly asymmetric blood separation matrix in conjunction with other porous media to perform clinical (not necessarily immunoassay) testing.
(WO) 9610177	Spectral Diagnostics, Inc. JJ Scharstuhl	04 APR 96	A 3-part diagnostic device comprised of a blood separating porous matrix, an immunochromatographic matrix, and a volume absorbing matrix (collector membrane) to control the volume.
(WO) 9603194	Pall Corporation RL Manteuffell	02 AUG 96	Porous composite materials useful as filtration media, especially for the separation of plasma or serum from blood.

What you should know before you specify a membrane for your next assay.

One of the most important components of an immunochromatographic assay is the lateral flow membrane.

This publication contains invaluable information that will help you understand the key considerations for specifying an optimal membrane for your next assay.

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