Guide

Rapid diagnostic test development: focus on lateral-flow assays

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cytiva

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Introduction

In former times, the need for diagnosis was intrinsically tied to diseases. People went to see the doctor when they were suffering from symptoms, and diagnostic assessments were performed to define diseases and decide on therapies. However, we've since switched from this old, symptom-based disease management to disease prediction and prevention. We've also seen diagnostic testing expanding from only human clinical tests to applications in areas such as veterinary medicine, agriculture, and even food and beverage production. This growth has been made possible by the increasing capabilities of diagnostics as well as the successful transfer of diagnostic technologies to point-of-care use. One of the most widely used analytical technologies in diagnostics is the immunoassay, which is now firmly established alongside more traditional, instrument-based techniques like ELISA, immunohistochemistry, and flow cytometry. Commonly used immunoassays take the form of lateral-flow, flow-through, dipstick, dry chemistry, or line-blot assays.

Cytiva manufactures high-quality products and has a presence in diagnostics component and service supply spanning decades. Our membranes and our cellulose and glass fiber materials have been used in immunochromatographic devices since the early beginnings of this technology. Now, our portfolio of products offers a broad range of membranes with properties such as high capillary flow rates and high protein binding capacities. As a single-source vendor, Cytiva offers a wide range of membrane, glass, and paper-based products.

This guidebook was created as an aide to help newcomers to lateral flow assay development. This overview, combined with the troubleshooting section, makes this book also a valuable resource of information for the experienced diagnostics assay developer and producer.

Lateral-flow immunoassays

In a lateral-flow immunoassay, a sample is driven by capillary forces through a test device past lines of immobilized capture molecules. The capture molecules then form complexes with detector molecules in the sample when they pass the detector molecules. Many variations of this principle are possible. An immunoassay needs several functions that are typically realized with different components (Fig 1). Usually a sample pad, a conjugate release pad, a reaction membrane, and an absorbent pad (or wick) are needed. Applications using whole blood require specific blood separation filters.

Within the lateral-flow format, there are two standard types of assays. Tests for macromolecular analytes (e.g., for the pregnancy hormone hCG) are typically sandwich assays, while tests for small molecules (such as drugs of abuse) are competitive or inhibition assays (Fig 2). The following sections will describe assay design and material selection in detail, including both of these test formats.

The reaction membrane

One of the most critical components of any lateral-flow immunoassay is the reaction membrane. The dynamics and kinetics of the assay are mainly defined by its protein binding and capillary flow characteristics, which in turn depend on the physical and chemical properties of the membrane.

The functions of the membrane are to immobilize the capture molecules at the test and the control line (typically antibodies) in a way that their binding properties for their respective target molecule are preserved while also guiding the flow of the sample and the detection conjugate to the reaction area. To do all of this, the membrane must have a high and consistent protein binding capacity as well as porosity and wettability to allow a capillary flow of the aqueous sample.

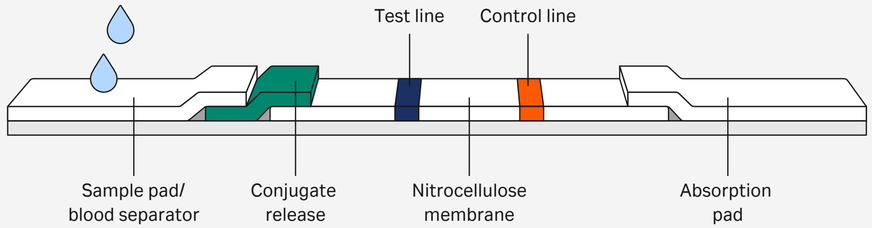


Fig 1. Typical assembly of a lateral flow immunoassay

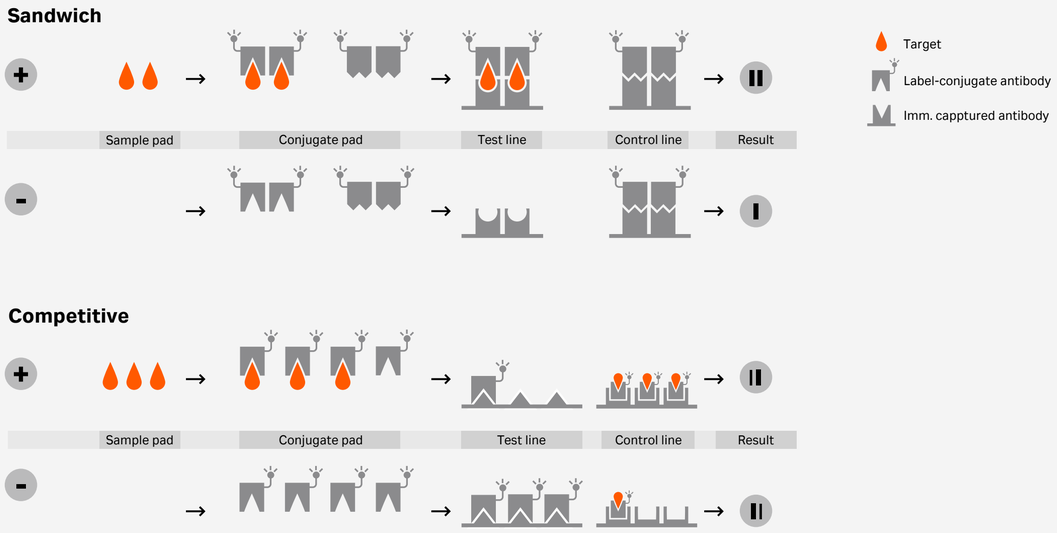


Fig 2. Sandwich-type lateral-flow assays are typically used for macromolecular analytes because at least two epitopes are required. Positive tests show two lines, and negative tests show only one (the control line). Competitive assays can be used for small molecule analytes that do not have two independent antibody binding sites. Instead of a capture antibody, a competitor (e.g., an analogue of the analyte) is immobilized. When analyte molecules are present in the sample, the binding sites of the detection antibody will be occupied, and the conjugate will not bind at the test line (i.e., analyte and competitor compete for binding to the conjugate).

A variety of polymeric, microporous membranes have characteristics that fulfill some or all the desired requirements, however, nitrocellulose (NC) is the polymer widely accepted as the most suitable membrane for most applications. Although nylon and polyvinylidene fluoride (PVDF) have similar protein binding capacities, these materials have drawbacks that make them less usable for immunochromatographic assays. Protein binding is more difficult and less reliable with both materials, and PVDF needs to be prewetted with methanol. As a result, nitrocellulose has been adopted for the vast majority of lateral-flow applications.

Protein binding forces

Achieving a high and consistent binding of active capture molecules to the test and control lines is a key element in production of a sensitive and reliable immunoassay.

Two reasonable models have been proposed to explain this protein-binding. The first model suggests that proteins are initially attracted to the membrane surface by electrostatic interaction, while long-term attachment is accomplished by a combination of hydrogen bonding and hydrophobic interactions. Although extremely difficult to prove, this model fits the published experimental data and is often the accepted mode of action (1–5). A second model suggests that the initial attachment of the protein is caused by hydrophobic interactions, with long-term binding by electrostatic forces. This model is also in agreement with much of the published data, however, the electrostatic partition mechanism may not provide a full explanation for the long-term stability conferred on protein attachment by drying or the use of an alcohol fixation step (6). As nitrocellulose polymers have no basic or acidic groups, effects of pH on protein binding are due to changes of the properties of the proteins and not of those of the membrane.

Whatever the combination of forces responsible for protein binding are, test developers should take all of them into consideration when seeking to optimize protein binding to an NC membrane.

Protein binding capacity

Independent of the molecular binding mechanism, binding capacity of a membrane is determined by the surface area of the polymer that is available for protein binding. The approximate surface area for membranes of different pore sizes can be estimated by looking at the surface area ratio (SAR) of each material. SAR represents the ratio of available surface area in the pores of the membrane to the area of the membrane used (Table 1). SAR depends on the thickness, pore size, and porosity of the membrane. It's obvious that inner surface area will increase linearly with thickness due to an increase in volume. The dependence of pore size and inner surface area is inversely proportional to the square root of the pore size, which means a 10 µm pore membrane has an internal surface area 10 times smaller than a 0.1 µm pore membrane. The internal surface area also increases nonlinearly with porosity. Porosity is the fraction of membrane that is not filled with polymer material (i.e., the space filled with air in the dry membrane). It can be also described as the void volume of the membrane. Pore size and porosity are largely independent parameters. A membrane can have large pores but a low porosity if the number of pores is low. Pore size is better referred to as "nominal pore size" because the value depends on the method used to determine it, and different manufacturers use different methods to do so. Thus, two membranes with the same nominal pore size can differ considerably when compared using a constant technique (Fig 3).

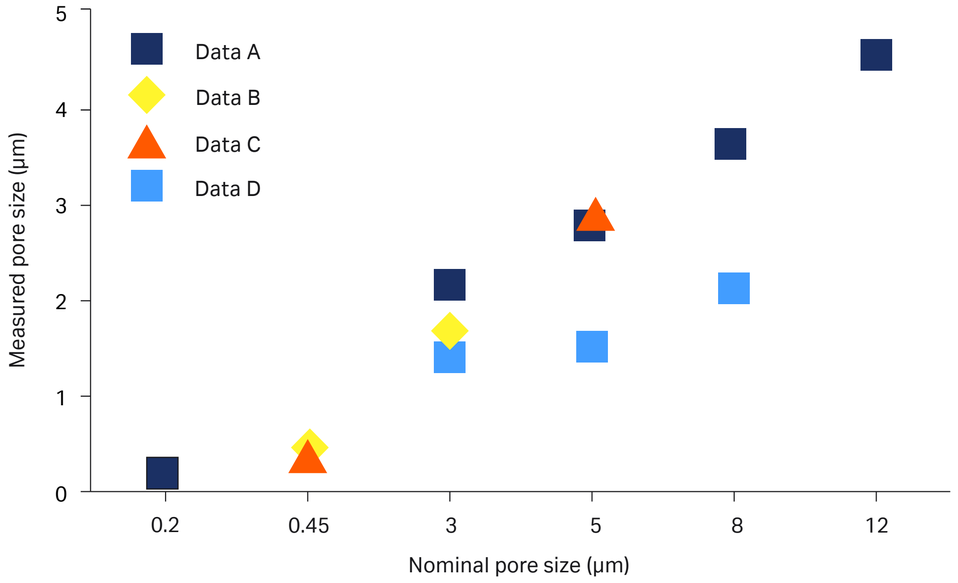


Fig 3. Membranes with the same nominal pore size can differ considerably when compared using a constant technique. All measured pore sizes for this comparison were determined using the same instrument, a Coulter porometer. Data A to data D are nominal pore sizes from different membrane manufacturers.

The amount of a protein that can bind to a given surface area depends on the size of the protein. According to various sources, an IgG molecule can be regarded as being a lens-shaped spheroid with a diameter of about 15 nm and a thickness of about 3 nm. For binding at random orientation, the maximum amount of IgG that can bind in a monolayer to a surface will be approximately 0.4 µg/cm^2 (7). As molecular weight and volume are proportional for globular proteins, the maximum amount that can bind will be similar for other proteins. With a SAR between 63 and 110, a test line of 1 mm width and 1 cm length (= 0.1 cm^2) can bind between 2.52 µg and 4.4 µg of IgG. This amount is much more than usually required for the mass of capture antibodies in an assay.

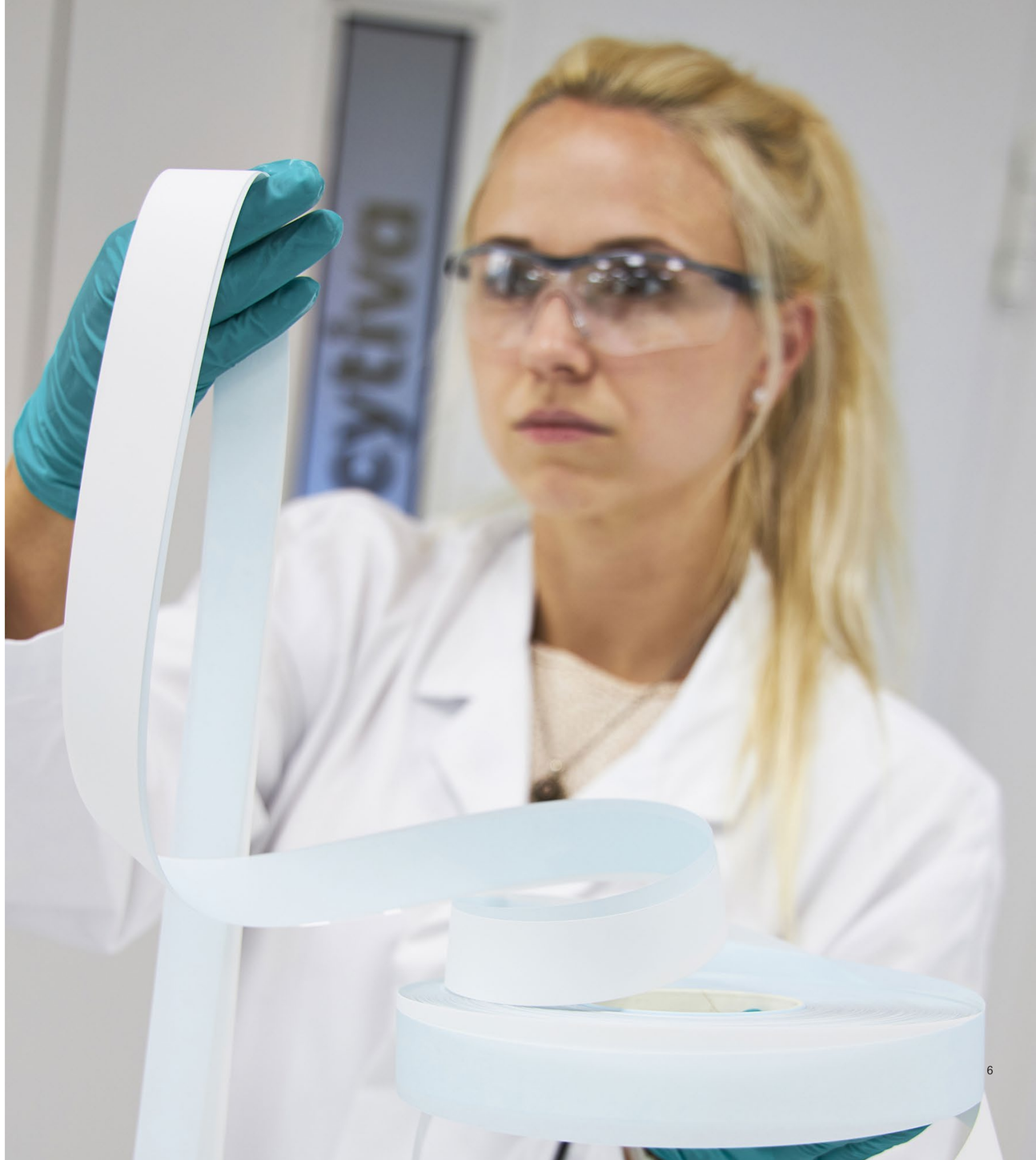
Methods such as using a Biacore™ for SPR Analysis allow you to understand the kinetics of your antibody pairs and suitability or limitations for incorporation into your lateral-flow assay.

Watch our webinar

Characterization and selection of antibodies using surface plasmon resonance (SPR) technology.

Table 1. Example surface area ratio data for Cytiva NC membranes. Data produced by BET surface area measurements using nitrogen.

Nominal pore size (µm)	**Surface area ratio** (SAR)
3	110
5	98
8	66
45	96



Capillary flow

The capillary flow rate is important because it not only defines the time needed to perform the assay but also the overall dynamics and sensitivity of the assay. In membranes of identical chemical composition, the capillary flow rate depends on the three-dimensional structure, namely pore size, pore size distribution, and porosity. With the exception of true pores membranes like our Nuclepore™ and Cyclopore™ track-etched membranes, membranes do not have discrete, well defined pores. A brief description of the process of membrane manufacturing will help to understand how the complex, microporous structure of an NC membrane is formed and how this can be controlled.

Membrane physics

In literature, membranes are usually defined by pore size and capillary rise time. As previously described, the nominal pore size depends on the method used for its determination. The particle retention method uses the permeation of particles of known size through the membrane. For this method, the pore size is the diameter of the largest particles that can pass through the membrane. The particle retention method is impractical for a routine check. Instead, the so-called “bubble point” is usually measured. The bubble point is the pressure (in bars) required to force air through the bottom of a membrane that has a layer of liquid on top of it. The larger the pore size is, the lower the pressure needed. This relationship can then be used to evaluate the pore size of a membrane. Both methods, particle retention and bubble point, have two weaknesses. They only determine the pore size in the flow-through direction and not in the lateral-flow direction (which are not necessarily identical), and they cannot be used for membranes cast directly on a backing film. For backed membranes, pore sizes can only be inferred from analogy with unbacked membranes or measured from SEM images.

The larger the pore size is, the higher the lateral-flow speed or wicking rate of the membranes (Table 2). This flow speed is measured as capillary rise time and decreases nonlinearly with the distance traveled (Fig 4), for example if the liquid front needs x seconds to migrate 1 cm, it will need 2x seconds for the next 1 cm. Hence, it is necessary to give the wicking rate as the time needed to migrate a defined distance.

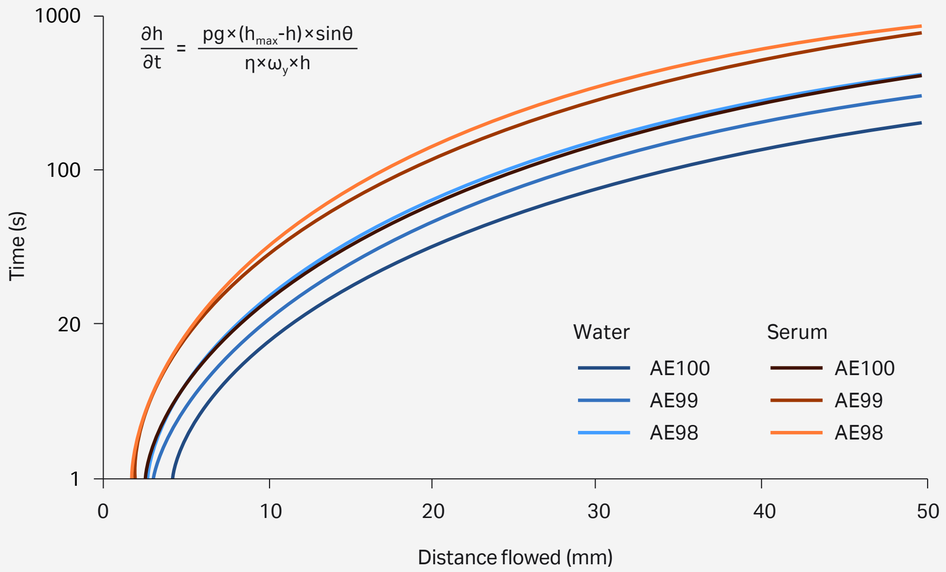


Fig 4. Capillary flow rates of Cytiva membranes of the AE family. The flow speed decreases nonlinearly with the distance the liquid has already traveled.

Table 2. Typical wicking rate data for Cytiva membranes. The tests were performed using water as the test liquid at 25°C and 49% RH.

Nominal pore size (µm)	Time to rise 4.5 cm (s)
3	245 ±35
5	185 ±25
8	140 ±20
12	100 ±20

Flow rate and assay kinetics

The flow rate of a membrane used in an immunoassay is one of the key influences on test results. For a given combination of membrane, capture reagent, and analyte concentration, the sensitivity of the test will increase with decreasing flow rate. In effect, the apparent concentration of the analyte increases as the flow rate decreases (Fig 5).

The relationship between the lateral wicking rate of a membrane and the apparent analyte concentration follows an inverse square law:

$$\text{Apparent analyte concentration } \mu \ 1/(\text{lateral wicking rate})^2$$

A doubling in the lateral wicking rate will reduce the apparent analyte concentration to 25% of the previous value. The explanation for this effect is simple: because analyte and capture antibody have only half of the time close enough to each other for binding to occur, the effective concentrations of both are halved. Because both contribute equally to the formation of the complex, the effect on apparent analyte concentration (i.e., formation of the complex at the capture line) is the product $0.5 \times 0.5 = 0.25$. Thus, as a general guideline, a developer who needs an assay with the ultimate sensitivity should select the membrane with the smallest possible nominal pore size.

Direct cast membranes

As NC membranes are rather fragile, the low tensile strength can be problematic for test production, especially when using reel-to-reel machines. To enhance the mechanical strength for lateral-flow applications, membranes have been developed that are directly cast onto a nonporous film. This film should not be too thick so that the membrane can still be provided in a roll format and to avoid the “memory effect” of curling when unrolled and cut. The backing film also makes the membrane practically unbreakable during normal processing steps in test production. An additional advantage is that the backing prevents direct contact between the NC material and the adhesive from the lamination card on which the elements of the test are mounted. Migration of components of the adhesive, especially volatile solvents, can have significant impacts on the properties of the membrane. By absorption of solvent vapors, the membrane may become more hydrophobic (8).

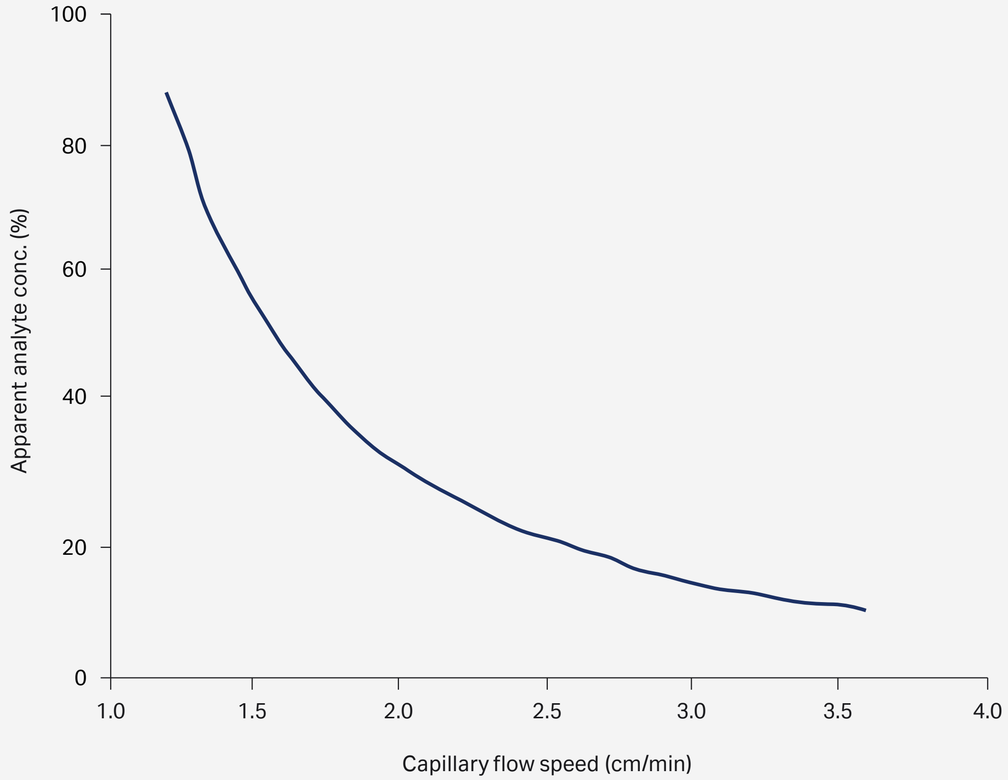


Fig 5. Assay sensitivity decreases with increasing capillary flow rate. The calculation of the curve is based on a flow rate of 1.2 cm/min = 100% assay sensitivity.

Backing films are typically polyester (PE) or polyethylenterephthalate (PET), and Cytiva backed membranes are cast on 100 µm (4 mil) polyester films. (The thickness of these films is often measured in mil and 1 mil = 0.001 inch). Backed membranes have one disadvantage: only the air side of the membrane is accessible. Due to the casting process, all NC membranes have some asymmetry between belt side and air side (Fig 6). Usually, the belt side is denser with smaller pores compared to the air side. When looking at the belt side of a membrane made by casting on a steel belt, the belt side is shinier because the surface is smoother and less likely to have irregularities. With an unbacked membrane, assay developers can test if one side is more suitable for their assay than the other, which, along with generally having a lower cost, is the advantage of unbacked membranes.

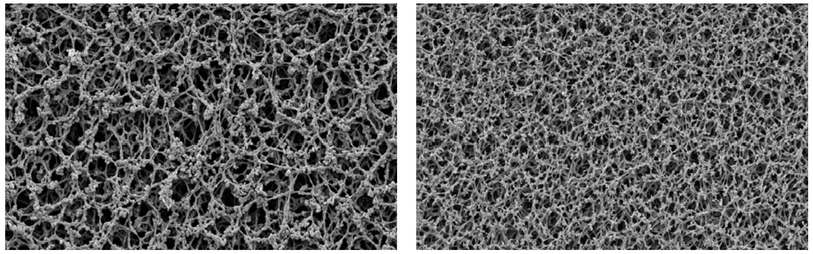


Fig 6. SEM images of a Cytiva AE 100 membrane at 500× magnification. Left image: air side; right image: belt side. The asymmetry is more marked in membranes with large pore sizes such as the AE 100 which has a nominal pore size of 12 µm.

Post-treatments

Following manufacture, nitrocellulose membranes routinely receive post-treatment processes to remove dust (unincorporated polymer left on the surface of the membrane after manufacture) or to modify their rewetting characteristics.

Advantages of post-treatment in membrane production

The industry at large generally accepts the practice of post-treating NC membranes to preserve their wetting properties. However, there is disagreement about whether membranes should be treated with a wetting agent before they are delivered to the customer or later in the manufacturing process after the capture line has been applied to the membrane. Purchasing membranes that have already been treated can be an attractive alternative, as it can reduce the amount of processing that the test manufacturer must perform. Such post-treated membranes can be used directly off the shelf, thereby eliminating the costs of additional equipment to perform a treatment step after protein application and the time required to do so.

Possible problems with post-treated membranes

One major disadvantage of using an off-the-shelf post-treated membrane is that the wetting agent can leach off or migrate through the membrane. These results can become especially noticeable when the membrane is stored for an extended period before use. Changes in the concentration of the wetting agent can affect the protein binding properties of the membrane as well as its wetting properties and lateral wicking rate. The shelf life of a test can thus become dependent on the concentration of wetting agent applied to the membrane. Because the wetting agent could possibly be applied some significant time before the test is actually manufactured, its concentration could be unknown when the membrane is used for test production.

On the other hand, when the manufacturer performs such post-treatment in-house after purchase, the assay developer can record the level of rewetting agent in the membrane and can conduct adequate aging studies on the material. This knowledge enables the developer to create appropriate post-treatment protocols that optimize the long-term storage and use of the membrane.

In untreated NC membranes, the hydrophilicity of the material is a direct function of its pore structure. But when such membranes are post-treated with a hydrophilic agent, it is the post-treatment that governs the hydrophilicity of the material. If the post-treatment migrates during storage or is washed off by the sample, the comparative performance of membranes with the same nominal pore specifications can change quite dramatically. Initial quality control (QC) testing can enable the manufacturer to determine the combined effect of the membrane's pore structure and hydrophilic post-treatment. But as the post-treatment ages or is removed, the performance of the membrane will become increasingly dependent upon its pore structure, and the results of the initial QC tests will become invalid. Product developers can ensure a less-variable product by using membranes from a supplier whose pore structure is consistent between manufacturing batches and membranes that have not undergone post-treatment with hydrophilic agents. The Whatman™ range of nitrocellulose membranes has both these advantages.

Because most post-treatment agents are water soluble, any water present when the capture line is applied can wash the post-treatment away from the point of capture line application. This washing away can result in a portion of the membrane lacking wetting agent and therefore becoming highly hydrophobic, which makes the capture line inaccessible to the sample and conjugate. These effects can significantly affect the readability of the assay. Frequently, such hydrophobicity causes the sample to pass unevenly through the capture line, resulting in striations or intensity variations (Fig 7). In extreme situations, the capture line can appear white against a colored background. Applying post-treatments after capture line application can avoid these pitfalls.

Fig 7. Water present during the application of post-treatments can make sections of the membrane hydrophobic, resulting in striations or intensity variations in the capture line. Both pre- and post-treated membranes are available from Cytiva.

Selecting a membrane

As discussed previously, nitrocellulose membranes are a key functional part of lateral-flow assays. The membrane must provide sufficient protein binding to enable the production of a sharp and intense capture line while keeping the level of nonspecific background low for easy interpretation of results. Capillary rise time defines the dynamics and kinetics of the lateral-flow assay, and the correlation of assay sensitivity and flow rate follows an inverse square law. A developer who needs an assay with the ultimate sensitivity should select the membrane with the smallest possible nominal pore size. Larger pore sizes will result in wider test lines because the capture reagent solution will diffuse quicker from the point of application into the membrane. The faster the wicking rate is, the wider the test lines will be.

Assay developers should weigh the time and cost benefits of using an off-the-shelf post-treated material against the consistency and long-term stability advantages of a material that contains no surfactant post-treatment.

Unsupported NC membranes are very fragile and may be difficult to handle. Their lack of elasticity and low tensile strength can make them unusable in some manufacturing workflows. Supported (direct-cast) membranes are easier to handle in test line application, lamination, cutting, and test assembly. The backing also prevents migration of adhesives into the membrane. However, with direct-cast membranes, the belt side is not accessible. In some assays, the belt side may show superior performance. With an unbacked membrane, assay developers can test if one side is more suitable for their assay than the other.

Whatman™ membrane properties

Table 3. Membrane properties and uses

Membrane family*	Membrane type	Description of grade	When to use	Product name	Size	Product code	Capillary rise (s/4 cm)	Total caliper (µm)	Format
AE	Unbacked	Constructed of 100% nitrocellulose with no treatments post manufacture, the AE family of membranes offers a level of purity and performance that cannot be seen in posttreated materials. As a result, AE membranes have been extensively used in the lateral-flow industry for the past 30 years.	AE membranes are unsupported, which means either the belt or air side of the membranes can be used, but the membranes are more difficult to handle.	AE98	25 mm × 50 m	10549916	160 to 210	120	Reel
				AE99	25 mm × 50 m	10548081	120 to 160	120	Reel
FFHP membranes	Backed	Our FFHP membranes were developed to have "faster flow and higher performance" than other membranes available at the time of launch. The membrane has a total thickness of 200 µm including backing and includes reduced surfactant content.	Quantificative assays or assays where you can reduce reagent dispensing to save cost on reagents. Designed for lateral-flow arrays.	FF80HP	25 mm × 50 m	10547003	60 to 100	200	Reel
				FF120HP	25 mm × 50 m	10547001	90 to 150	200	Reel
				FF170HP	25 mm × 50 m	10547005	140 to 200	200	Reel
FFHP Plus membranes	Backed	A variant on our FFHP membrane containing a higher concentration of surfactant and a different surfactant to overcome hydrophobic issues. 200 µm thick membrane including backing.	When using viscous samples and you wish to reduce reagent dispensing rates to save cost.	FF80HP Plus	25 mm × 5 0m	10547042	60 to 100	200	Reel
				FF120HP Plus	25 mm × 50 m	10547040	90 to 150	200	Reel
				FF170HP Plus	25 mm × 50 m	10547044	140 to 200	200	Reel

*Membranes laminated on self-adhesive backings also available upon equiry.

Membrane family*	Membrane type	Description of grade	When to use	Product name	Size	Product code	Capillary rise (s/4 cm)	Total caliper (µm)	Format
FFHP Plus Thick membranes	Backed	A thicker version of our FFHP Plus membrane. 235 µm thick including backing.	Optimized for easy swap-out or secondary supply for a 235 µm thick membrane.	FF80HP Plus Thick	25 mm × 100 m	10547156	60 to 100	200	Reel
				FF120HP Plus Thick	25 mm × 100 m	10547149	90 to 150	235	Reel
				FF170HP Plus Thick	25 mm × 100 m	10547147	140 to 200	235	Reel
Vivid™ LFNC membrane	Backed	A thicker membrane that is post-treated, providing alternative options in development	Optimized for easy swap-out or secondary supply for a 235 µm thick membrane.	Vivid 70 LNFC	25 mm × 50 m	40069680	50 to 90	190 to 230†	1 roll
				Vivid 90 LNFC	25 mm × 50 m	49904266	70 to 110	190 to 230†	1 roll
				Vivid 120 LNFC	25 mm × 50 m	49904173	95 to 135	190 to 230†	1 roll
				Vivid 140 LNFC	25 mm × 50 m	40069675	120 to 160	190 to 230†	1 roll
				Vivid 180 LNFC	25 mm × 50 m	40069677	160 to 200	190 to 230†	1 roll
Immunopore™ membranes	Backed	Immunopore is the membrane of choice for producing the most sensitive assays. This membrane, available in three wicking rates, is directly cast onto a plastic backing and offers unparalleled sensitivity.	The Immunopore range provides the best solution for anyone working with critical assays (such as for infectious disease) whose reproducibility, stability, and accuracy are paramount.	Immunopore RP	25 mm × 50 m	78356403	90 to 140	200	Reel
				Immunopore FP	25 mm × 50 m	78336403	140 to 200	200	Reel
				Immunopore SP	25 mm × 50 m	78316404	190 to 280	200	Reel

*Membranes laminated on self-adhesive backings also available upon equiry. †Include 95–105 µm polyester support.

Sample pad, sample preparation, and absorbent media sample

Wick

The function of a sample pad is to accept the sample and transport it in a homogeneous and consistent manner to the conjugate release area or analytical membrane depending on how the test is configured. One of its functions is to ensure that the test is not flooded with sample. The sample pad can fulfill many additional tasks. It can be used to filter particulates or cells out of the sample, and it can be impregnated with chemicals to modify the sample. In many cases, the sample pad is also the ideal place to add blocking substances.

A sample pad is typically made of a wettable material that should be essentially inert. Most importantly, the analyte in question should not bind to the pad material. Suitable materials can be cellulose, glass fiber, and woven polymers.

Sample preparation

Samples can have undesired properties that can be influenced by additives in the sample pad. Some sample types show a broad sample-to-sample variation. For example, urine samples differ considerably in pH and ionic strength and molecular composition. Impregnation of the sample pad with salts and buffers can make samples more similar when they reach the conjugate release and test zones. In other cases, an addition of a substance making the sample more viscous may be helpful to reduce the wicking rate and enhance the sensitivity of detection.

Blocking agents (generic proteins such as BSA and/or detergents such as Tween 20) are often needed to avoid nonspecific binding of the analyte or detection reagents to the reaction membrane. Efficient blocking will increase the signal-to-noise ratio (e.g., visibility of the test lines by reducing background stain). The addition of blocking agents to the sample pad and conjugate release pad, respectively, is often much easier and also more cost efficient than adding them to the reaction membrane. This method is often called “blocking on the fly.”

However, addition of blocking agents to the conjugate pad can cause problems due to the interaction of blocking agents and conjugate. Hence, the sample pad can be the best place to deposit the blocking agent.

Whole blood separation

Blood serum is one of the most interesting sources for important diagnostic markers. For a rapid test, especially when intended for bedside or field use, the classical methods of serum or plasma preparation are not practical. There is an increasing demand for whole-blood assays with separation of blood cells within the test device.

The separation of blood cells from plasma using a filter is a demanding task. The percentage of particulates (mainly erythrocytes, the contribution of leukocytes is negligible), and the hematocrit or packed cell volume (PCV) is between 36% and 48% for females and between 40% and 53% for males. Peak values can be up to 70% (typically found in blood of Tour de France participants). These values mean that a droplet of blood from a finger stick does not contain much liquid. It is common for lateral-flow immunoassays with whole-blood samples to add some reagent to the sample.

Adding reagent to a blood sample ensures that there is sufficient liquid volume to perform the assay. It is also advisable to add an anti-coagulant to this reagent to avoid clotting problems. In the presence of such an additive, the effective sample in the assay is plasma, not serum.

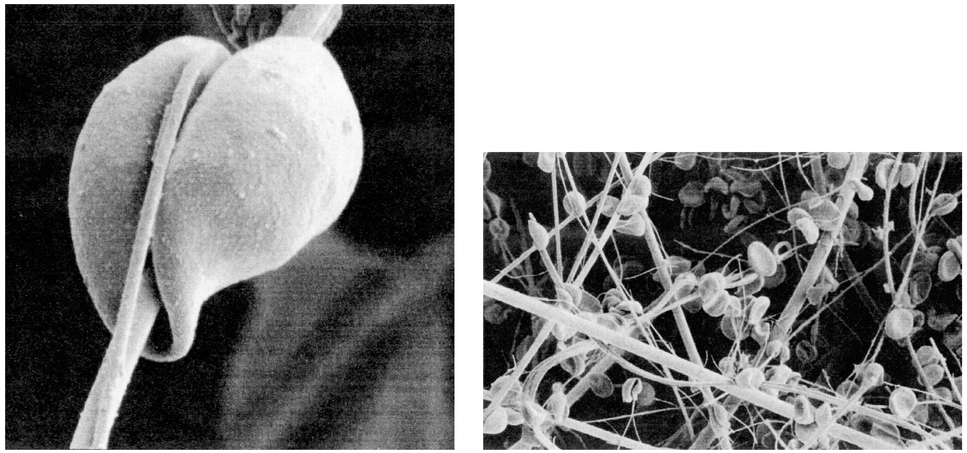


Fig 8. Scanning electron micrograph of blood cells trapped on the fibers of a glass filter.

A blood separator for immunochromatographic assays is typically a cellulose or glass fiber depth filter (Fig 8). In addition to efficiently removing blood cells, the separator must also yield a high recovery of plasma from the sample, must not be hemolytic, and should not interact with the analyte in question. Larger blood volumes call for thicker filter pads, but a higher void volume will also reduce the recovery of serum (Fig 9). Hemolysis, the breakdown of red blood cells, can have significant effects on diagnostic tests. Even if only 0.5% of the erythrocytes of a blood sample are lysed, the resulting serum or plasma will have a clearly visible pinkish color. This color can increase the background of an immunoassay. Other constituents of the cells can directly influence the results of the assay (e.g., erythrocytes contain a much higher concentration of potassium ions than blood plasma). Hemolysis can also change the plasma levels of clinically relevant markers such as lactate dehydrogenase, alkaline phosphatase, kreatine kinase, and troponine T (to name some of the more prominent markers). Whatman blood separators do not lead to a considerably higher degree of hemolysis than traditional methods of preparing blood serum (Fig 10).

Absorbent pad

In a lateral-flow assay, the absorbent pad or wick is the component that is located on the end opposite of the assay from the sample application zone. Its primary function is to act as a sink for the reagents. If a lateral-flow device does not have an absorbent pad, the flow stops as soon as the sample front has reached the distal end of the reaction membrane. In this case, the sample volume that can be analyzed is defined by the void volume of the membrane (i.e., membrane length × width × porosity). Adding an absorbent pad can substantially increase this volume.

A typical effect in lateral-flow assays is that the direction of flow reverses when the membrane starts to dry after the assay has ended. The liquid that had been collected by the absorbent pad starts to act as the source. This backflow effect often causes false positive results when the assay is read too late. Whatman grade CF6 greatly reduces this effect. CF6 is a glass fiber and cellulose blend material with very high water absorbance and fast flow rate.

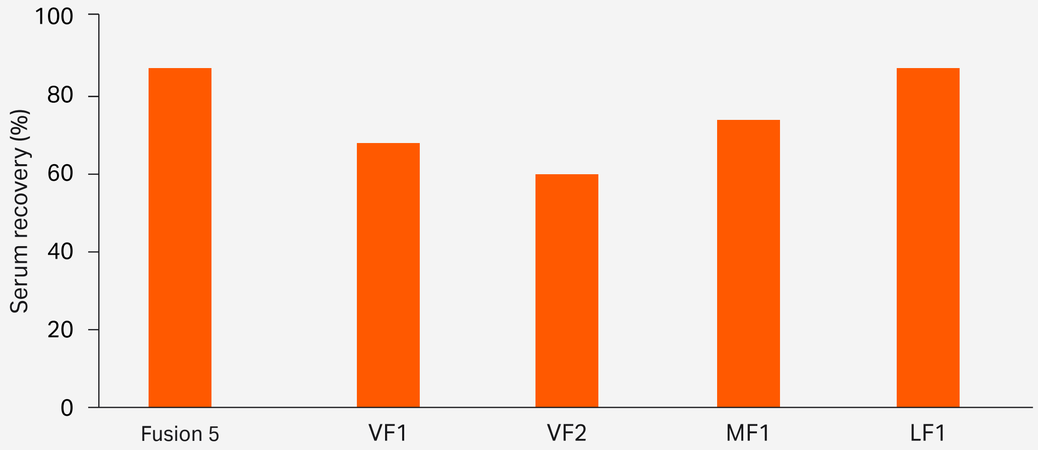


Fig 9. Typical percentage of available serum obtained from a whole-blood sample of 40 µL with different blood separators.

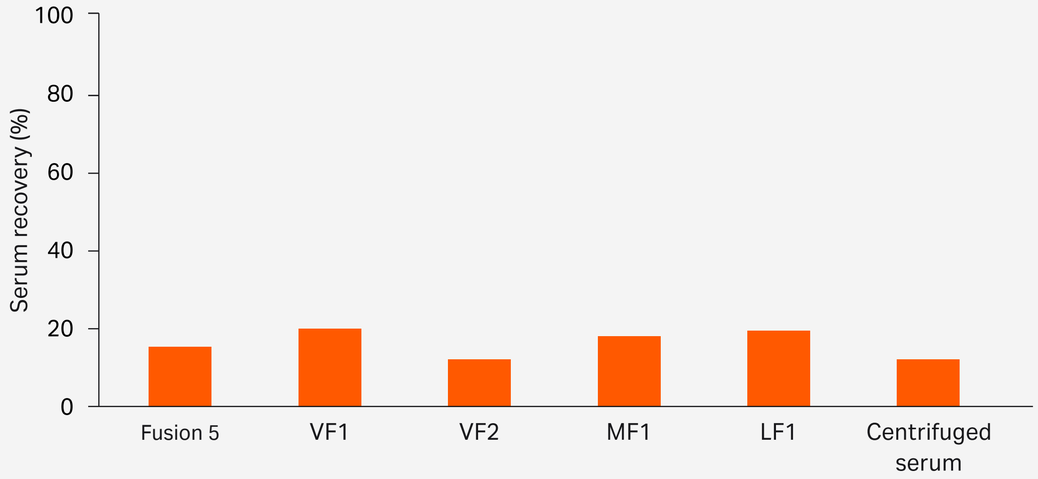


Fig 10. Free hemoglobin in serum recovered from blood separators or prepared by centrifugation from clotted blood.

Selecting sample pad and absorbent media

Parameters that are often taken into consideration when choosing a material for the sample pad are physical properties such as basis weight and caliper, water absorbance, and wicking rate. The larger the volume of sample that must be accepted by the sample pad, the higher the bed volume of the pad must be. The wet strength of the material is mainly important when paper rolls must be pulled through a bath for impregnation.

The main function of the absorbent pad is to act as a sink. It must be made of a hydrophilic material with high water uptake and high volume. Such a material is typically a cellulose material. Another important consideration is material handling during the production of the test (cutting, lamination, etc.). Once a specific material is selected, the liquid volume uptake can be modified by changing the length of the pad. Whatman offers several grades of pure cellulose made from 100% cotton linters (Table 5). Grade CF6 is a blend of cellulose and glass fiber. If CF6 is used as an absorbent pad, there is less chance of the conjugate running back down the strip after the end of the assay. This unique performance makes it the ideal choice when conjugate backflow is a concern.

Cytiva offers a variety of cellulose and glass fiber materials as well as cellulose-glass fiber blends to ensure that all sample types and specific needs can be addressed (Table 4). CF1 is a lightweight grade for small sample volumes. CF3, CF4, CF5, and 470 are medium-weight grades that can accept medium and large volumes. CF7 and 300 are very thick grades for high sample volumes. Standard 14 and 17 are glass fiber materials that offer faster flow and higher sample yield than cellulose. Rapid 24 and 27 are treated glass fiber materials especially treated for optimized conjugate release. GF/AVA and GF/DVA are bound glass fiber filters that are especially suitable when small particles must be removed from the sample. This property makes them the first choice for saliva samples.

Another option when you want high volumes of plasma recovery without lysis is to use an asymmetric membrane such as Vivid plasma separation media. The asymmetric nature of the membrane allows the cellular components of the blood (red cells, white cells, and platelets) to be captured in the larger pore sizes, while the plasma flows down into the smaller pores on the downstream side of the membrane.

Table 4. Suitability of Cytiva cellulose and glass fiber materials as wicks for different sample types

Grade	Aqueous samples	Urine	Saliva	Nasal swabs	Whole blood	Serum
CF1, CF3, CF4	+	+				+
470, CF10	+	+				
Standard 14 and 17	+	+				+
Rapid 24 and 27	+	+				+
GF/DVA	+		+	+		
LF1, MF1, VF1, VF2					+	
Fusion 5	+	+			+	+

Sample pad and absorbent media properties

A lateral-flow assay begins with the sample pad transporting the sample from its point of application to the downstream test components. The absorbent pad at the distal end of the test controls sample flow along the strip. To ensure that your assay begins without complications, Cytiva offers a complete range of high-quality sample pad materials. Cytiva has also developed absorbent media with excellent wicking characteristics that give rise to greater consistencies.

Most of the grades now used as components in lateral-flow tests were originally used for various analytical applications such as filtration or chromatography purposes. These special grades have been designed to have specific characteristics and are well specified and controlled for diagnostic applications.

Table 5. Properties of sample and absorbent pad materials

Grade	Properties	Thickness (µm at 53 kPA)	Wicking rate (s/4 cm)	Water absorption (mg/cm^2)
100% cotton linter				
CF1	A thin, smooth-surfaced cotton linter paper with a liner flow rate, suitable for small volumes.	176	187	16
CF2	A thin, smooth-surfaced paper containing an FDA approved resin that binds the cellulose fibers together. Suitable for small volumes.	172		16.1
CF3	A medium thick cotton linter paper, originally used for separation of inorganic compounds. Larger sample volume than CF1.	322	174.3	34.6
CF4	A medium thick cotton linter paper with acid treatment to improve wet strength and reduce trace impurity content. Similar weight and thickness to CF3 with faster wicking.	482	67.3	49.9
CF5	100% cotton linter for medium to high volumes	954	63.3	99.2
CF7	100% cotton linter for medium to high volumes	1873	35	252.3
CF10	A medium thick cotton linter paper with similar absorbency to CF4, for medium volumes.	490		42

Grade	Properties	Thickness (µm at 53 kPA)	Wicking rate (s/4 cm)	Water absorption (mg/cm^2)
Glass fiber and cotton				
CF6	Glass fiber and cellulose mix. Higher absorbency than CF5, lower absorbency than CF7. Solves conjugate backflow issues.	1370	65	128
Glass fiber – bound glass fiber, treated and bound glass fiber				
VF2	Vertical separator used as single or multiple layers for separation.	785	23.8	86.2
GF/DVA	Untreated bound glass fiber.	785	28.2	93
MF1	Untreated bound glass fiber suitable for whole blood or serum. Typically used for whole blood volumes between 30 µL and 100 µL.	367	29.7	39.4
LF1	Untreated bound glass fiber suitable for whole blood or serum. Performs well with one or two drops of whole blood.	247	35.6	25.3
GR470	Untreated bound glass fiber suitable for whole blood or serum.	840	77	78
Standard 14	Untreated bound glass fiber for faster flow than cotton with lower sample retention. Higher absorption capacity than Standard 17	355	42	55
Standard 17	Untreated bound glass fiber for faster flow than cotton with lower sample retention. Greater tensile strength than Standard 14.	370	47	35
Rapid 24	Treated bound glass fiber for good rewetting of membranes with higher absorption capacity than Rapid 27. Optimized as conjugate release material.	340	38	55
Rapid 27	Treated bound glass fiber for good rewetting of membranes with greater tensile strength than Rapid 24. Optimized as conjugate release material.	365	39	40
Single layer matrix performing all functions of a lateral-flow strip				
Fusion 5	A proprietary, single-layer matrix membrane that can perform all of the functions of a lateral-flow strip. It can be used as sample pad as well as a blood separator with excellent separation efficiency.	370	43.9	42.3

Table 6. Materials for rapid separation of whole blood in lateral-flow tests

Product grade	Minimum blood volume (µL)	Separator area (cm^2)*	Serum recovered from 40 µL blood	Basis weight (g/m^2)	Thickness (µm at 53 kPA)
LF1	20	0.59	21.2	46	247
MF1	20	0.77	17.6	57	367
Fusion 5	20	0.63	21.5	56	370

* Minimum area of separator to separate the minimum volume

Conjugate release pad

Detection reagents in lateral-flow immunoassays are typically antibodies conjugated to colloidal gold particles, latex beads with visible or fluorescent dyes, or paramagnetic beads. The function of the conjugate release pad is to accept these reagents, keep them in a functional state during the shelf life of the product, and to release them when the sample moves through the pad. The pad also needs to have good flow characteristics to enable a uniform and reliable transfer of sample and conjugate to the reaction membrane.

The conjugate is typically applied in a buffer containing stabilizers to maintain the binding properties of the antibody and to facilitate resolubilization. The buffer may comprise surfactants, sugars, stabilizing proteins, and hydrophilic synthetic polymers. The pad can be impregnated with the conjugate, or the conjugate can be sprayed or striped onto the pad. In case of impregnation, a consistent bed volume of the pad material is needed to minimize variation of the quantity of conjugate in the final pad.

Release of the conjugate should be efficient. Conjugate that remains on the pad does not contribute to the signal. Hence, adsorption of the conjugate to the pad should be perfectly reversible. The conjugate pad should also not interact with the sample; in particular, it shouldn't bind the analyte. Conjugate release pads must be hydrophilic. Typical materials used are glass fibers, surface-modified polyesters, and rayons. Glass is hydrophilic by nature, and, in contrast to synthetics, it does not need surface modifications to render it hydrophilic. Polyesters and rayons typically show larger batch-to-batch variation. Glass fibers show better consistency and are more reliable for test production. In addition to the standard grades, Cytiva offers glass fiber materials that are specifically treated to enhance conjugate release (Fig 11).

Conjugate release is a key player in the overall performance of the assay. The kinetics of conjugate release determines how much of the analyte in the sample can be measured. When the liquid front of the sample migrates through the conjugate release pad, it starts to dissolve the detection reagent. If the conjugate has completely left the conjugate pad before the last analyte molecules have entered it, these cannot contribute to the signal. Analyte molecules reaching the test line later than the detection reagents can still bind to the capture molecules, but there is no conjugate to form the sandwich complex. Thus, the sample volume actually analyzed by the assay is not necessarily the same as the volume applied to the assay but is instead the volume needed to completely release the conjugate. In an optimal assay, these volumes are identical. For these volumes to be equal, the amount of conjugate should be adjusted to match solubilization by the sample.

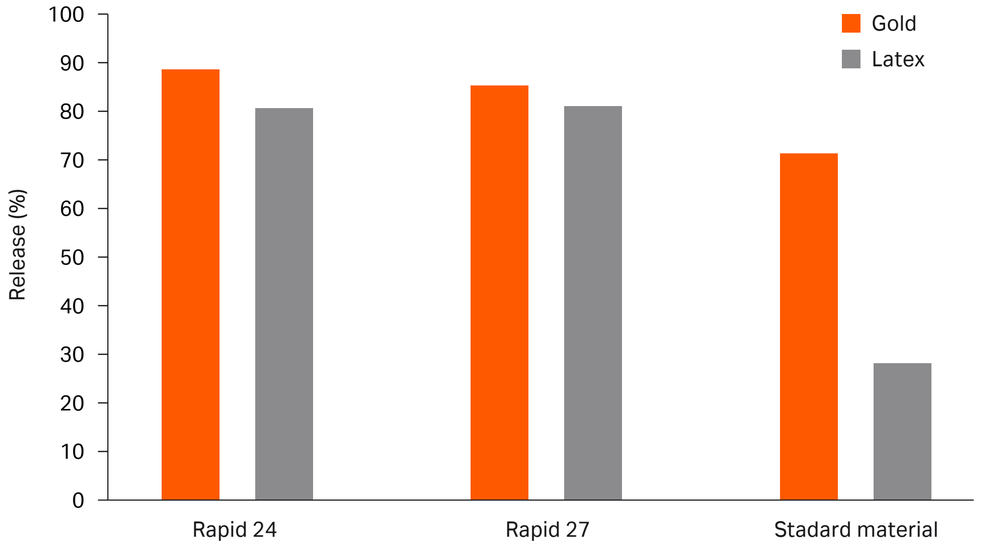


Fig 11. Release of antibody conjugates from pads of treated and untreated glass fiber material. Determination after 90 s. Rapid 27 and 24 enhance the release of colloidal gold protein conjugates but are even more beneficial compared to untreated, standard material when using latex beads.

Selecting a conjugate release pad

Cytiva offers a range of glass fiber materials and nonwoven media for conjugate release, each with different absorption and release properties. Glass fibers are naturally hydrophilic. Hence, surfactants and other release-facilitating agents are not needed when applying the conjugate. However, to maximize release and minimize aging effects, assay developers often use additives. The rapid release range was developed to offer materials that can be used without the need of further treatments. Alternatively, a test developer can select another media and treat it with blocking agents or use separate conjugate release agents to suit exactly the requirements of an individual assay system. The rapid range allows an “off the shelf” product to be used for conjugate release, although the product is designed to give better average performance across a wide range of tests rather than optimal performance for any particular assay. Careful optimization of another grade for a specific assay may result in improved performance.

Conjugate release properties

Conjugate release pads are critical to lateral-flow assays. To ensure consistent performance and a strong test line, the conjugate must dry without damage or aggregation and release rapidly when the sample is applied. For consistent, reliable results, diagnostic manufacturers rely on Cytiva products. Cytiva conjugate release pads made of glass fiber do not require treatment prior to conjugate application, as they are inherently hydrophilic. The open structure of the material allows rapid penetration by both conjugate and sample. Cytiva nonwoven conjugate release pads made of synthetic fibers are very fast and resistant to many common solvents and detergents. Their relatively small caliper also makes them a popular choice for prefiltration applications.

Table 7. Properties of conjugate release pad materials

Grade	Properties	Thickness (µm at 53 kPA)	Water absorption (mg/cm^2)	Percent release of gold conjugate (after 90 s)
Standard 14	Untreated bound glass fiber for faster flow than cotton with lower sample retention. Higher absorption capacity than Standard 17.	355	55	75
Standard 17	Untreated bound glass fiber for faster flow than cotton with lower sample retention. Greater tensile strength than Standard 14.	370	35	75
Single layer matrix performing all functions of a lateral-flow strip				
Fusion 5	Fusion 5 can perform all the functions of a lateral-flow strip. It can be used as a sample pad as well as a blood separator with excellent separation efficiency. As conjugate release pad, it offers excellent release with both latex and gold conjugates.	370	42.3	> 94

Grade	Properties	Thickness (µm at 53 kPA)	Water absorption (mg/cm^2)	Percent release of gold conjugate (after 90 s)
Treated bound glass fiber: optimized for conjugate release				
Rapid 24	Treated bound glass fiber for good rewetting of membranes with higher absorption capacity than Rapid 27.	340	55	89
Rapid 27	Treated bound glass fiber for good rewetting of membranes with greater tensile strength than Rapid 24.	365	35	86
Accuflow G	Treated bound glass fiber with high volume capacity that allows it to be used as a sample pad as well as for conjugate release.	370	40	89
Nonwoven media (treated)				
16 S	Non-woven media with high tensile strength	420	22	
Asymmetric polysulfone membrane				
Vivid PSM GF	Specifically engineered for the generation of plasma from whole blood	330 ± 20	20	N/A
Vivid PSM GR		330 ± 20	20	N/A
Vivid PSM GX		330 ± 20	20	N/A

Other components of lateral-flow immunoassays: detection conjugates, backings, and housings

Common conjugate labels are colloidal gold, colloidal carbon, latex beads with visible or fluorescent dyes, and paramagnetic beads. The choice between these options is typically driven by a combination of requirements. Fluorescent and paramagnetic beads maybe the first choice when developing a reader-based test. Availability of different coupling chemistries and the possibility of covalent and/or site-directed coupling is also a benefit of latex particles. The pros for colloidal gold are mainly ease of use, high reproducibility, and lower cost. Paramagnetic particles require a reader, however, a reader-based system using these detection conjugates can yield excellent quantitative data and extreme sensitivity.

There are several suppliers specializing in detection reagents for immunoassays. For gold colloids, there exist also a number of protocols to make home-brew conjugates. These protocols are based on the reduction of HAuCl4 to form gold atoms. The protocols are generally quite simple but making monodisperse colloids of uniform size and shape with minimal variations requires chemicals of very high quality. Performing the protocols require much experience, too. Particles of irregular shape and size will coat unevenly with protein during conjugation. Irregular particles may not repel each other in solution, which will lead to aggregate formation and problems with lateral flow in the assay. Low quality gold conjugates are also much more likely to give false assay results.

Fig 12. A well-made 40 nm gold colloid shows a typical cherry-red color.

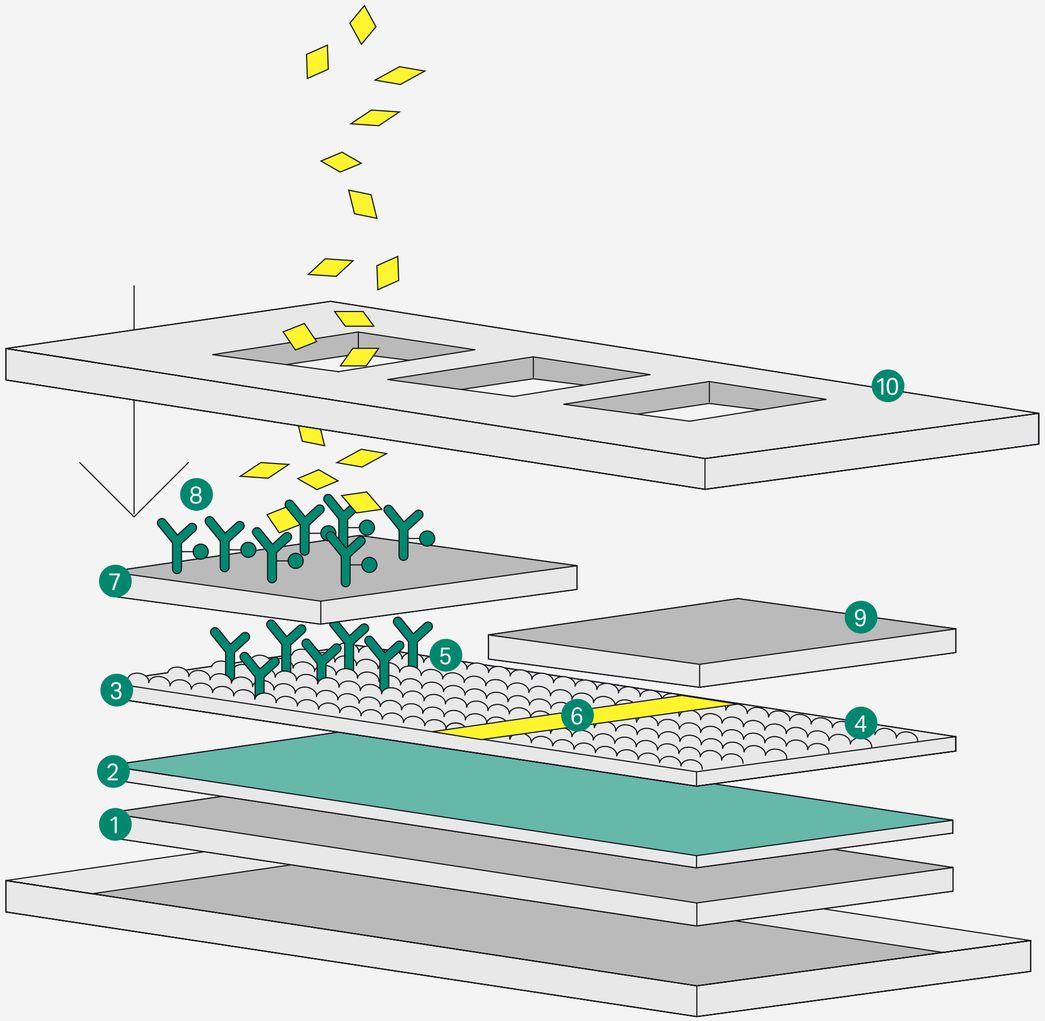


Fig 13. Expanded view of a lateral flow immunoassay test device. 1 = backing, 2 = backing adhesive, 3 = reaction membrane, 4 = blocking reagent, 5 = capture antibodies, 6 = control line, 7 = conjugate release pad, 8 = conjugate, 9 = absorbent pad, 10 = housing

The choice of backing and adhesives is also critical. You can purchase cards similar to a double-sided tape from which you peel away a protective seal to expose the adhesive and attach your components. The adhesive can show chemical incompatibility with components of the assay, especially after long term storage and when unbacked reaction membranes are used (8). Hence, a medical grade adhesive should be used or, at minimum, an adhesive whose solvent base is released very slowly over time and is low in silicon. The adhesive layer must have sufficient thickness and fluidity to ensure mechanical stability of the test assembly but must not disperse into the test components or interfere with the cutting process into the final strip format of the individual test device. The plastic backing should give the test device the necessary mechanical strength. Mechanical strength is usually higher for test formats without housing such as a dipstick test. Too thick a backing can result in production problems such as issues with the cutting process or making the test assembly too rigid to be rolled. A number of backing materials designed for lateral-flow test production is commercially available.

The design of the housing of a lateral-flow immunoassay is a critical part of assay development particularly when creating a reader-based system. The reading instrument may introduce specific requirements due to optical and/or mechanical constraints. Light reflective properties or autofluorescence of the material can be problematic. You may also wish to protect the instrument from liquid leaking out of the assay cassette. These constraints should be taken into account from the beginning of assay development. Generally, the assay housing must match the dimensions of the assay device. The view window must match the position of the test lines on the reaction membrane and the sample application window must be suitable for the sample volume to be applied. The cassette should apply suitable pressure onto the test components to ensure good contact and reliable flow. Excess pressure can have obstructive effects.

Creating and optimizing a lateral-flow immunoassay

A lateral-flow immunoassay is a simple device for the end user. However, the more it is designed to be easy to use and give fast and reliable results, the more complex the tasks of development and production of such a device can be. A lateral-flow immunoassay consists of multiple components and uses multiple reagents. There are many parameters that all influence one another to a degree. Slightly changing one assay component can change the entire assay. To make developing your assay easier, we suggest that you take into consideration the following ideas:

- Creating a test that can be 100% accurate in the shortest possible time with the smallest sample size may not be realistic. It may be helpful to set flexible requirements for your system. Thinking in terms of ranges is often the best approach.
- It may be necessary to increase sample size or go with slower flow rate to achieve optimal accuracy and reliability. Sometimes it helps to choose the single parameter that is most important and then experiment with the other assay components and reagents to finally reach a satisfactory compromise within the requirements.
- It is necessary to take into consideration that each reagent put into the system may affect the system as a whole. If, for example, you introduce a detergent to aid in flow rate, you may inadvertently decrease the sensitivity of the assay because this detergent may also interfere with protein binding.
- Similar compounds from different manufacturers are never identical. This fact is especially true for the reaction membranes. Different manufacturers use different raw materials, polymer make-ups, and wetting agents and use different methods to specify their products. If you change from one manufacturer's material to another, you may find that you need to adjust some assay parameters to make an optimal match with the different material.

Optimizing protein binding

In immunochromatographic assays, the primary function of a protein applied to a membrane is to act as a capture reagent for the target analyte in a sample. Because the test result is totally dependent upon achieving a good binding of the capture reagent to the membrane, the importance of achieving a high and consistent level of protein binding cannot be overstressed (Fig 14).

Four critical areas can affect protein binding to the NC membrane:

1. The application buffer in which the capture reagent is dissolved
2. The membrane itself
3. The capture reagent itself
4. The ambient conditions (mainly humidity) at the time of protein application

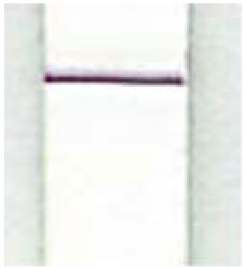


Fig 14. The aim of a lateral-flow test is to achieve a clear and crisp test line. Achieving this aim largely depends on correct binding of the capture reagent to the NC membrane.

Capture reagents

Capture reagents used in immunoassays are typically antibodies, mostly IgG. Despite this fact, no capture reagent is absolutely identical to any other. An optimization of binding is most straightforward with monoclonal antibodies. Optimization with polyclonal antibodies is likely to be more difficult due to the heterogeneity of the proteins. Each subpopulation may require slightly different conditions for optimal binding. Species such as IgA or IgM can present an even greater challenge because of the potential for structural or steric problems. Capture proteins other than antibodies can cause significant difficulties either because of their chemical properties or their size. Usually, protein binding to a surface is stronger for larger proteins.

Impurities of other proteins and carrier proteins present in the preparation will compete with the capture antibody for binding to the surface. Normally, the protein binding capacity of NC membranes exceeds what is needed to immobilize sufficient amounts of capture protein. However, the binding capacity may not be sufficient when the ratio of capture molecules in the preparation used to apply the test lines is low.

Effects of ambient humidity

Nitrocellulose membranes are very susceptible to ambient conditions. Dry conditions can render the membrane more hydrophobic. A very obvious effect of low ambient humidity on membranes is the buildup of a considerable static charge. A static charge can make unbacked membranes difficult to handle and make them attract dust particles that are almost impossible to remove without damaging the membrane.

Low humidity at the time of dispensing the capture reagents will have a significant effect on the resulting test line, especially when a noncontact application system is used. Water droplets naturally carry a negative charge and are repelled when sprayed on a membrane at low ambient humidity. This repulsion will generate “satellite spots.” In extreme cases, no continuous line will exist because the majority of droplets do not follow a straight path. Conversely, extremely high ambient humidity can result in very rapid wicking of the applied protein causing wide or diffuse capture lines.

Although most experienced assay developers will have their own optimal range of ambient humidity, 40% to 60% relative humidity at temperatures of 18°C to 22°C is generally a good range. Allow the membrane to equilibrate with the atmosphere before striping the capture lines onto it. The necessary equilibration time should be determined by experimental investigation.

Membrane effects

The pore size of a membrane defines the internal surface area that is available for protein binding and thus its overall binding capacity. The smaller the pore size, the higher the protein binding capacity. In addition, pore size can influence the appearance of the test lines because of the spread of the capture reagent after application. In a membrane with a high lateral flow rate, the protein will rapidly diffuse from the point of application causing a wider and more diffuse line. With all membranes, a part of the capture reagent does not contribute to the signal because it is bound in the depth of the membrane and isn't visible. Only dye conjugate captured within the top 10 µm layer of a NC membrane is visible. The percentage of capture protein penetrating deeper into the membrane is very likely to be larger in membranes with large pore size. This consideration is irrelevant when paramagnetic beads are used as detection reagents because any particle present in the test line region will contribute to the signal irrespective of its vertical position in the membrane.

For a series of membranes with a fixed surface area, the level of protein binding is a function of polymer type and the presence of any treatment agents that affect the surface energy of the membrane. The base polymer for membrane production is available from a number of commercial sources; but each source material has slightly different properties. Nitrocellulose is made by esterification of cellulose; hence, it is based on a natural product. In addition, a variety of different membrane treatments are used by membrane manufacturers. It is always advisable for product developers to conduct experiments to evaluate the relative protein binding performance of any membranes they are considering for their tests. The protein binding capacity range should also be included in the specifications for the membrane material used in production of the test device.

Application buffers

A suitable application buffer must solubilize the capture protein in the concentration needed for application and stabilize it (i.e., keep it in solution and preserve its binding properties). Achieving optimal binding may also mean creating conditions that make it energetically favorable for the protein to partition onto the solid phase. As capture proteins vary, optimal conditions for binding these proteins to the membrane may also be different.

It is essential that the protein is soluble in a suitable concentration for capture line application in the buffer. If the available concentration is too low, it will be difficult to apply sufficient capture protein to the line. If the protein forms precipitates, they can clog the pores of the membrane and cause an irregular flow of the sample through the test region of the strip. Precipitates will probably also cause problems with the application equipment by blocking tubes and orifices.

Although the use of destabilizing and coprecipitating agents can induce a state in which the partition of the protein onto the solid phase is energetically favored, it makes sense to start with finding a suitable solvent for the protein. Key properties that can be modified for optimization are pH and ionic strength.

The pH level

The pH of the application buffer can have a significant impact on protein binding to the membrane. The NC membrane has no acidic protons; hence, all pH effects are due to influences on properties of the proteins. Solubility of a protein is minimal at its isoelectric point (pI).

Extreme pH values can denature a protein and cause massive alterations of its binding properties or even formation of aggregates and precipitation. Following the rule to seek conditions favoring the partition from solution onto the solid phase, a pH at or close to the pI should be ideal for an application buffer for the test lines.

Popular buffer systems are phosphate, borate, carbonate, and TRIS. Keep in mind that, in a lateral-flow assay, the capture molecules are dried in the membrane after application. As a result, all buffer constituents will also be dried into the membrane unless they are volatile. Therefore, volatile buffers like ammonium acetate or ammonium carbonate are especially attractive. High concentrations of primary amines (e.g., from TRIS buffers) can result in formation of salt bridges between acidic amino acid residues (Glu, Asp) of the capture protein and the amino groups of the buffer ions. These salt bridges can mask binding sites.

Ionic strength

Solvation by ions in an electrolytic solution will decrease protein-protein attractive forces. Thus, solubility of a protein typically increases within a given range of ionic strength of the solvent (“salting-in” effect). At high ionic strength the effect reverses (“salting-out”). Now more and more water molecules get occupied for solvation of the ions and the solvation layers around the protein molecules collapse. Protein molecules can interact, form aggregates, and precipitate.

Following the rule that the protein should be soluble in the application buffer but kept under conditions that favor partitioning to the solid phase of the membrane, it may appear to be appropriate to utilize the salting-out effect to minimize molecular stability in solution. However, salts like ammonium sulfate may make the effect on destabilization of the protein solution difficult to control. Small variations of the salt concentration (e.g., by evaporation) can have severe effects on the degree of precipitation. In addition, using these types of buffers will introduce a large amount of salt into the membrane that can have severe effects on the assay after drying. We recommend reduced the salting-in effect by keeping the ionic strength of the buffer as low as possible. Buffered salines (PBS, TBS) are generally not recommended.

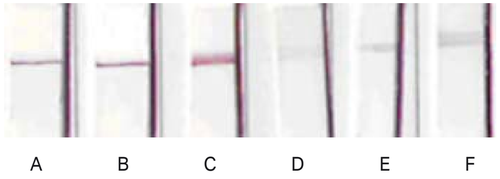


Fig 15. Varied results from capture lines of 1 mg/mL mouse IgG applied using different buffers: (A) 10 mmol phosphate, pH 7.2; (B) 10 mmol phosphate, + 3% methanol, pH 7.2; (C) 10 mmol phosphate + 150 mmol NaCl + 3% methanol, pH 7.2; (D) 50 mmol phosphate + 150 mmol NaCl + 1% BSA, pH 7.2; (E) 50 mmol phosphate + 150 mmol NaCl, pH 7.2; (F) 50 mmol phosphate + 150 mmol NaCl, pH 6.0. All samples were detected by a 40 nmol gold-conjugated goat antimouse IgG antibody.

Coprecipitating agents

When modifying an application buffer, developers may choose to add a destabilizing or coprecipitating agent to reduce the stability of the protein molecules in solution and facilitate binding to the solid phase. The action of such coprecipitating agents on antibodies relies on the differing stability that the fc and f(ab) regions of the IgG molecule have toward the agents used (5). The structure of the fc region is far more likely to be denatured by the action of coprecipitating agents. Partial destabilization of the fc regions leads to the exposure of more-hydrophobic groups that are normally hidden within the protein structure.

Thus, regardless of which mechanism is accepted for the binding of proteins to nitrocellulose, the increase in protein hydrophobicity resulting from the use of such coprecipitating agents will improve protein binding.

The most commonly used coprecipitating agent is alcohol (typically methanol, ethanol, or isopropanol), which can be recommended for a number of reasons. The presence of alcohol helps to rewet the membrane, reduces any static charge it may have, and has a destabilizing effect on the protein in solution. Alcohol also reduces viscosity and surface tension of the solution, which facilitates both dispensing and entry into the membrane. It also enhances drying. Levels of between 3% and 5% methanol can give considerable improvement in the performance of a membrane used for an immunoassay.

The use of alcohol to improve protein binding to a solid phase has been known for several years in the production of ELISA plates and is now regarded as a standard protocol (5, 9). The influence of aliphatic alcohols on binding in NC membranes was first reported in 1980, while a 1% isopropanol solution is widely used as a fixing solution in protein blotting experiments (6, 10).

Considering the points outlined above, a buffer comprised of 10 mmol phosphate + 3% methanol pH 7 is suggested for initial development studies. Although such a buffer will not prove optimal for all applications, it offers a very good starting point for the development process.

Optimizing conjugate deposition and release

To generate a signal in an immunoassay, a detection antibody is coupled to a signal reagent. Signal reagents can be enzymes (as in ELISA), dyes (mostly fluorochromes), colloidal gold, colloidal carbon, or latex particles. Lateral-flow immunoassays require a dense packaging of dye. Hence, typically colloidal gold or dyed latex particles are utilized. The use of colloidal gold is particularly well established. The small particle size and noncovalent mode of protein conjugation make gold colloids especially easy to work with. The amount of antibody needed for conjugation is comparatively low. On the other hand, the stability of gold conjugates is relatively low.

Surfactants and hydrophilic polymers present in the conjugate pad as blocking agents can interfere with protein binding to the gold particle to such an extent that the protein is finally detached. Blocking proteins can compete with the detection antibody for binding to the gold particles and displace the antibody if the proteins' affinity to the gold is greater. Latex beads are commercially available with different reactive groups on their surfaces thus offering different modes of covalent attachment of detection molecules. Some methods also enable a site-directed attachment. Beads with coatings for noncovalent but specific binding such as streptavidin and protein A are also available. Negative aspects of latex beads are their larger batch-to-batch variation, the higher amount of antibody needed for conjugation, and their larger size, which can cause aggregation problems in the reaction membrane.

As described earlier, in a sandwich type assay, the volume of sample required to completely release the conjugate is the portion of sample that contributes to the measured signal. On the other hand, conjugate that remains immobilized on the release pad when the sample passes through is not only wasted but will bind analyte molecules and reduce the test signal. To maximize sensitivity, conjugate release should be quantitative. In poorly optimized systems, conjugate release often decreases with storage time (Fig 16). The demanded shelf life of a finalized test device is typically 18 to 24 months. Within this period of time, aging effects can be significant if no methods are implemented to reduce them. Methods to improve conjugate pad performance can be coating with blocking agents and the use of separate release agents.

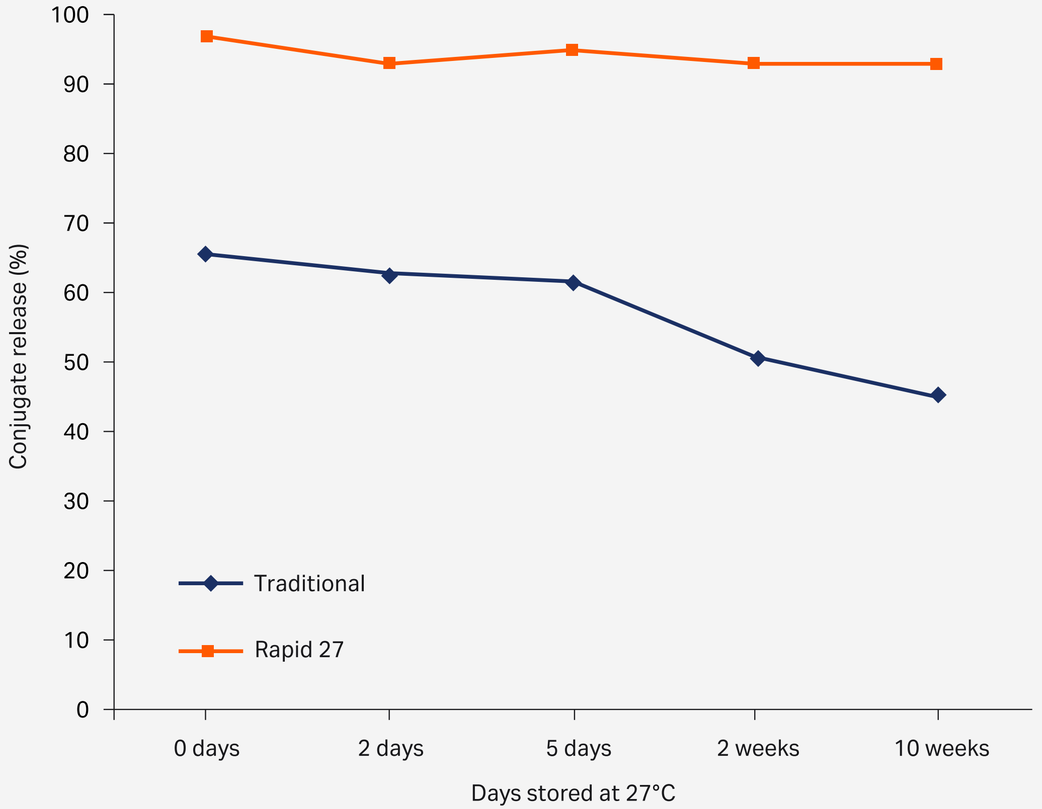


Fig 16. Efficiency of aging effects on treated and untreated conjugate release and conjugate release materials

Blocking agents

Coating of the conjugate release material with blocking agents is a typical method to improve performance. There are four types of materials that are traditionally used, either alone or in combination. These are surfactants, proteins, sugars, and synthetic polymers.

Detergents

These substances interfere with hydrophobic interactions and thus are very efficient release agents. Tween 20 and Triton X-100 are the most popular detergents. They are typically used in concentrations between 0.1% and 1.0%. However, they can have significant negative effects on antigen-antibody interaction in the test region (Fig 17). They can also reduce the stability of the conjugate when the detection protein is noncovalently attached as is the case with colloidal gold.

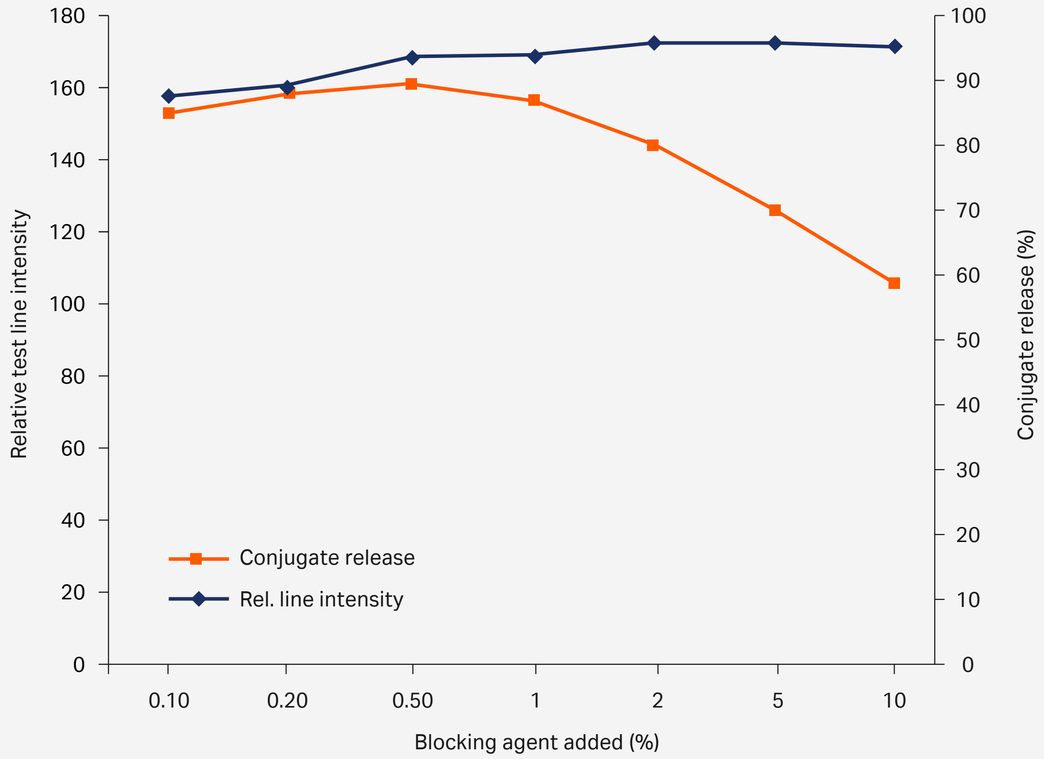


Fig 17. Tween 20 reduces the test signal when used in too high concentrations as a blocking agent on the conjugate release pad. Different detergents will show different curves. Anionic detergents (e.g., SDS) show a reducing effect on test line intensity at much lower concentrations.

Data shown for conjugate pad material Rapid 27, 40 nm colloidal gold particles coated with goat-anti-mouse AB.

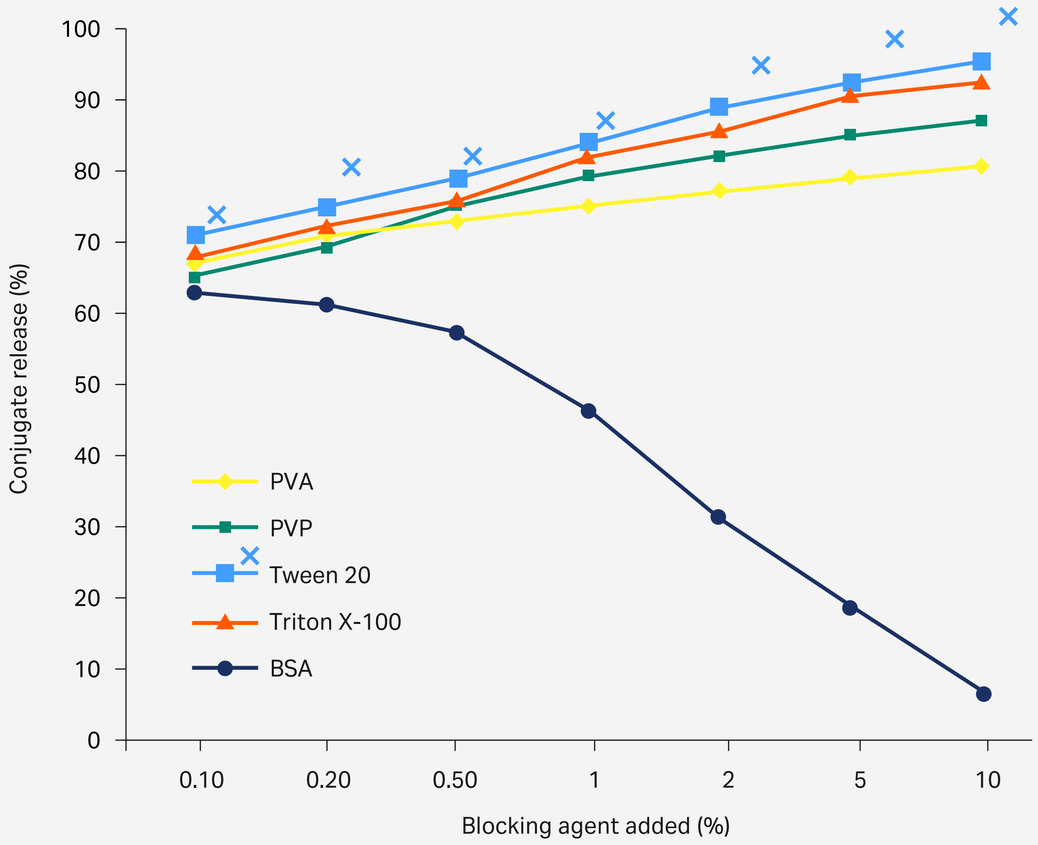


Fig 18. Effects of diverse blocking agents on release of a colloidal gold conjugate from Standard 17 material. The effect of BSA is reduction of conjugate release.

Proteins

Traditional blocking agents used in immunoassays are BSA and nonfat dry milk powder, which consists mainly of casein. They have been used for many years in a number of different applications including lateral-flow devices. Typical concentrations are 1% to 2%. Proteins are very efficient in reducing nonspecific background signals. However, bulk proteins can also have detrimental effects on conjugate release (Fig 18). The most likely explanation is that a high number of proteins in the conjugate release pad increases its hydrophobicity and reduces rewetting. Whether negative influences outweigh the positive effects of bulk proteins appears to depend on many factors and is difficult to predict.

Sugars

The effects when using sugars are ambiguous. Sugars are very unlikely to interfere directly with protein interaction with the pad matrix. They will probably only increase conjugate release efficiency when the fibers of the matrix are coated with sugar and the direct contact of conjugate and matrix is disabled, which requires high concentrations of the coating solution. Hence, a large amount of sugar will be solubilized by the sample, which will increase viscosity and reduce the lateral flow rate. These changes are normally undesirable. Low concentrations of sugar included in the conjugate application buffer are ineffective for conjugate release but may help to retain the protein structure and thus increase signal intensity due to improved binding to the analyte (11). Suitable concentrations are typically between 1% and 5% but much higher concentrations may be required in some cases.

Hydrophilic synthetic polymers

Polyvinyl pyrrolidone (PVP) and polyvinyl alcohol (PVA) are most frequently used. Pre-treatment with these polymers provides a hydrophilic surface on which to dry the conjugate. Typical concentrations used are between 0.5% and 2.0%. Low molecular weight polymers such as PVP K-30 are ideal as they do not increase the viscosity of the sample. A combination of a hydrophilic polymer and a detergent seems to be the most effective solution in many cases.

Their effects are complimentary, and a high degree of conjugate release is achieved at relatively low concentrations of the additives, which avoids problems that might arise if used at higher concentrations.

Separate release agents

The difference between release agents and blocking agents is that release agents are physically separate from the conjugate. They are deposited upstream of the conjugate release zone, typically in the sample pad. Alternatively, they can be applied to the upstream end of the conjugate release pad. Again, detergents and hydrophilic polymers are the most efficient release agents. A typical mixture is 1% PVP and 1% Tween 20 (this, of course, is subject to optimization).

Sugars and proteins are largely without effect as release agents. However, protein is often included for blocking on the fly of the membrane. The main advantage of using separate release agents is that a physical separation of release agent and conjugate avoids the problems that can occur by direct contact such as detaching of the antibody from the gold particles. However, coating of the conjugate release pad is usually more efficient.

Conjugate application techniques

There are two basic techniques for applying the conjugate to the release pad: impregnation and dispensing. Both are extensively used in rapid test production, and both have advantages and disadvantages. The choice is based upon personal experience, cost issues, and available machinery.

Impregnation

During impregnation, the conjugate release material is dipped into a solution of the conjugate and subsequently dried. To achieve consistent results, the material must be completely saturated in the solution. For large-scale production, immersing and drying can be performed with reel-to-reel machines equipped with suitable drying devices. However, the reduced tensile strength of the wet material may be problematic. Impregnation may not be compatible with coating the pad material with blocking agents before application of the conjugate because they can be washed out in the dip-impregnation process. Impregnation requires larger amounts of conjugate than dispensing does, which increases production costs. On the other hand, the large amount of conjugate deposited in the pad may also lead to stronger test lines. Removing the large volumes of solvent within a reasonable time without heat-damaging the conjugate can also be problematic. The major drawback of impregnation is low reproducibility due to thickness variation of the conjugate release material. This variation can lead to considerable concentration differences between individual test devices.

Dispensing

During dispensing, the conjugate solution is applied by an air-jet or through a nozzle. The volume deposited per centimeter of pad length can be precisely controlled, and the amount of conjugate can be easily optimized. This control avoids waste and reduces cost. Low liquid volumes enable rapid drying. Dispensing on coated material will normally not interfere with the coating. These obvious advantages make dispensing the method of choice for conjugate application for many test manufacturers. However, dispensing is not completely without problems. Insufficient conjugate may be introduced into the pad material because too much remains on the surface. Furthermore, spraying bears the risk of contaminating neighboring components.

Optimizing membrane flow

The flow rate of a membrane used in an immunoassay is one of the key influences on test results. For a given combination of membrane, capture reagent, and analyte concentration, the sensitivity of the test will increase with decreasing flow rate. The relationship between the lateral wicking rate of a membrane and the apparent analyte concentration follows an inverse square law. The flow rate of a membrane is mainly determined by the pore size but is greatly influenced by post-treatment. The flow in the assay also depends on sample properties such as its viscosity. The rate of rewetting and lateral flow of a membrane can be influenced by adding blocking reagents.

Table 8. Materials that show efficient membrane blocking properties.

Material	Typical working range (%)
Tween 20	0.01–2
Triton X-100	0.01–2
PVA (15 kDa)	0.1–5
PVP (33 kDa)	0.1–7
PEG (20 kDa)	0.05–3
Brij	0.05–3
BSA	0.01-3

When working with membranes that have been post-treated by the manufacturer with rewetting agents, it is normally not necessary to repeat this step. However, when rewetting and lateral flow are too slow or when nonspecific binding of hydrophobic analyte molecules causes a high background, blocking may be required. The purpose of blocking with detergents or hydrophilic polymers is to reduce hydrophobicity of the membrane. Reducing hydrophobicity should simultaneously minimize background staining and achieve even and consistent wetting upon sample application at a rate appropriate for the assay being developed. Table 8 lists some typical blocking agents and their working range. Again, a combination of a detergent and a hydrophilic polymer often provides the most desirable effect (Fig 19). Proteins such as BSA are very efficient in reducing the level of nonspecific background (Fig 20). However, they may also have adverse effects on the lateral flow rate of the membrane.

Blocking agents can also have negative effects. A substance that reduces nonspecific interactions of molecules is also very likely to reduce the desired interaction of analyte and capture reagent thus reducing the test signal. An increase of the capillary flow rate will in turn also reduce the apparent analyte concentration.

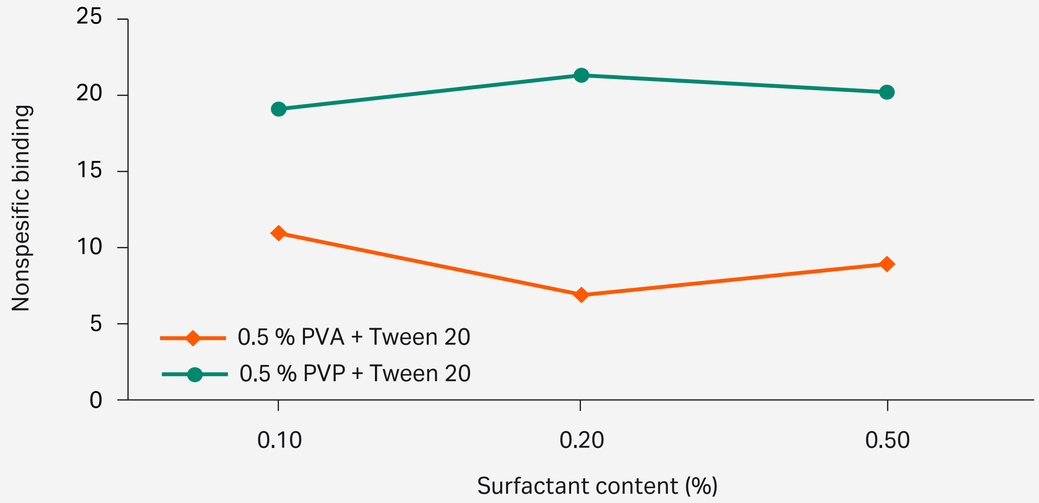


Fig 19. Effects of combinations of a detergent and a hydrophilic polymer on nonspecific background staining.

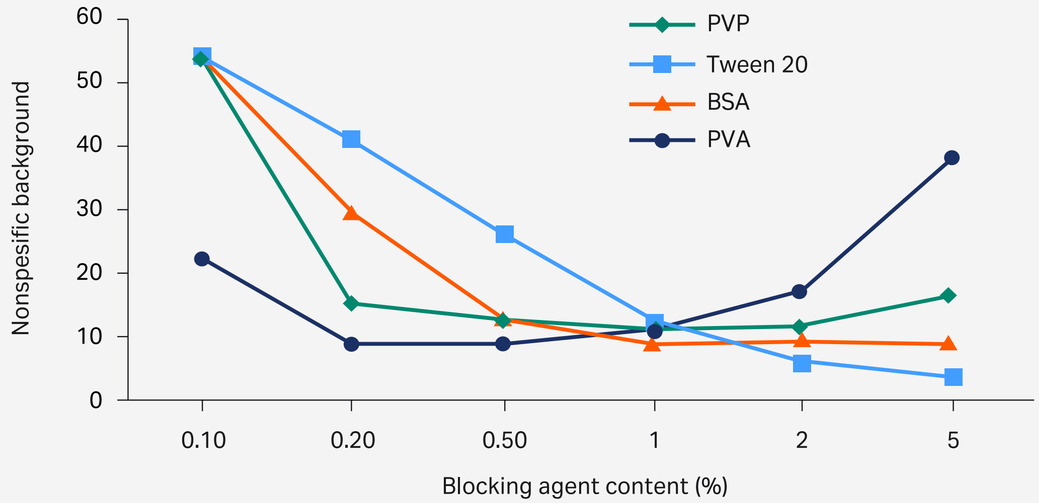


Fig 20. Effects of single detergents or hydrophilic polymers on nonspecific background staining.

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