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Lateral Flow Tests

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I. INTRODUCTION

Once limited to testing labs, lateral flow test (LFT) technology has now come of age in the post-pandemic world. At the onset of the pandemic, viral detection methods relied on either plate culturing or real-time PCR (Polymerase Chain Reaction) applications that required time, trained personnel, and sophisticated equipment to perform. Although these methods are still gold standards in the industry, the use of rapid, point-of-care testing was in demand to facilitate early identification of SARS-CoV-2. Although LFTs were already available for pregnancy testing over the counter (OTC), it was not until the pandemic that LFTs became commonly recognized throughout the world.

The very features that made LFTs an integral part of the pandemic response still are relevant to expand the reach of healthcare from developed centers with experts to areas of the world with limited access to supplies, trained experts, or facilities to perform testing. LFTs have:

- **User-friendly formats** that support at-home use without significant training.
- **Quick results** provide essential information to users and their healthcare providers whether located near or far.
- **Long-term stability** for use in different climates and shipping conditions.
- **Inexpensive construction** to manufacture for reduction of healthcare costs.

But developing an LFT is not as easy as one would expect based on their simplicity of use. There are essential parameters to ensure LFTs function as expected. In 2019, the World Health Organization (WHO) established a set of criteria for tests, which is described by the acronym, REASSURED (Land *et al*., 2019).

- Real-time connectivity
- Ease of sample collection and environmental friendliness
- Affordable
- Sensitive
- User-friendly
- Rapid and robust
- Equipment-free
- Deliverable to end users

LFTs are the type of tests that satisfy the WHO criteria, and therefore, there are tests for diagnostic applications in humans and animals, environmental monitoring, agricultural applications (Anfossi *et al*., 2010; Kim *et al*., 2019; O'Farrell, 2015), and food safety screening (Song *et al*., 2014). Since tests are performed in different environments, the lateral flow format is ideal. With proper preparation and storage, no refrigeration or special handling is required.

II. LATERAL FLOW TESTING TECHNOLOGY OVERVIEW

LFTs, also known as rapid diagnostic tests, lateral flow assays, lateral flow immunoassays, strip tests, dip tests, or immunochromatographic tests, are an extension of latex agglutination tests developed by Singer and Plotz in 1956. Agglutination testing, like LFTs, relies on the interaction of an antigen or analyte and its specific antibody, where the antigen/analyte is a biomarker for a particular infectious disease, medical condition, or phenotypic characteristic that is present in a sample of blood, urine, saliva, etc. For the recent pandemic, most LFTs were designed to identify the SARS-CoV-2 nucleocapsid protein, which is found in a sample from the nasopharyngeal region. But LFTs do not need to only identify a specific protein, they can also detect nucleic acids, whole cells, small molecules, or even something as small as an atom.

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The antibody half of the interaction needs to be specific to the antigen, function in the analyte, and flow through the lateral flow device. These are essential to the specificity and sensitivity of your test, so key parameters of the antibody and detection reagents are expanded below.

The LFT has multiple components that provide the correct environment for the assay to occur (Figure 1). The external cassette houses the supporting adhesive card with the attached sample pad (4), conjugate pad (3), membrane (1), and absorbent pad/wicking pad (2). The external cassette has the sample port and multiple windows to see the test line(s) and control line(s) which are printed on the membrane. To assemble the test:

1. The conjugate pad is loaded with visibly dyed microspheres, fluorescent microspheres, or colloidal gold conjugated to an antibody specific to the antigen/analyte.
2. The test line (TL) is created by coating a portion of nitrocellulose membrane with a substance that captures either the beads bound to the antigen/analyte or the beads that are not bound to the antigen/analyte. The different options are described below.
3. The control line (CL) is created by coating a portion of nitrocellulose membrane with an antibody that recognizes the bead or colloidal gold whether it is bound by antigen or not.
4. The sample flows through the sample pad and conjugate pad where it encounters the antibody complexed to the chosen microparticle (colloidal gold, visibly dyed microsphere, or fluorescent microsphere).
5. If there are analytes or antigens in the sample, they will bind to the antibody on the chosen microparticle. These complexes travel through the membrane next, where they may or may not bind to the TL, depending on the reaction scheme.
6. The chosen microparticles continue to travel to the CL, where they bind whether the analyte/antigen is present or absent.

Tests that function properly will always react with the CL. The presence or absence of the biomarker is determined by the reaction at the TL. The reaction mechanisms for both lines vary from one LFT type to the next, and new variations are continuously being developed. In the next section, we will outline a few reaction schemes used with microparticles.

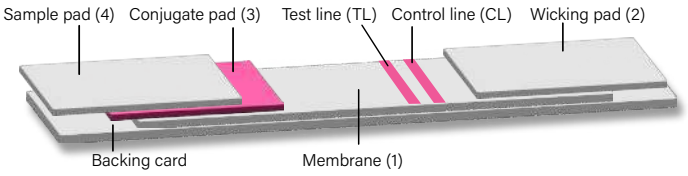


Figure 1 Lateral Flow Device Components

III. REACTION SCHEMES

The two predominant approaches to the tests are the Noncompetitive (or direct) and Competitive (or competitive inhibition) reaction schemes. These can best be explained graphically, as shown in Figure 2 and Figure 3.

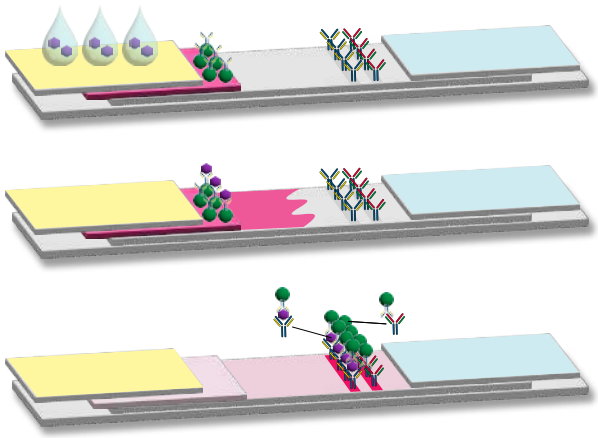
A. Direct or Noncompetitive Tests

The double antibody sandwich assay format is used when testing for larger analytes with multiple antigenic sites, such as luteinizing hormone (LH), human chorionic gonadotropin (hCG), or human immunodeficiency virus (HIV). In the direct test, the amount of antibody bound to dyed microsphere beads or colloidal gold should be more than the analyte/antigen found in the sample. In this type of test,

- Dyed beads or colloidal gold are conjugated to Antibody 1, which is specific for the biomarker antigen/analyte being tested. These are dried into the conjugate pad.
- The TL is coated with Antibody 2, which also is specific for the biomarker being tested (but to a different epitope).
- The CL is coated with Antibody 3, which recognizes the Antibody 1 whether it is bound to an antigen or not, typically, species-specific anti-immunoglobulin antibodies, specific for the conjugate antibodies on the microspheres.

A positive test result has a visible reaction at both the TL and CL. A negative test result has only a reaction at the CL.

A. Positive Test



B. Negative Test

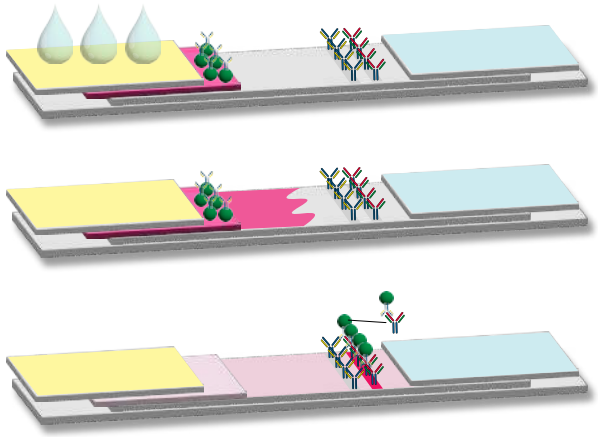


Figure 2 Direct or noncompetitive lateral flow assay. (A) When the sample droplets have analytes or antigens recognized by antibody 1, the bead:antibody:antigen complexes attach to antibody 2 in the test line (TL) and antibody 3 at the control line. (B) Because antibody 2 only binds to the antigen in the sample, a negative test will not have a signal at the TL. In contrast, antibody 3 binds to the bead:antibody 1 conjugate whether or not it is bound to an antigen, ensuring that the control line will be visible with negative and positive samples.

B. Competitive Reaction Scheme

The competitive reaction scheme is used most often when testing for small molecules with single antigenic determinants, which cannot bind to two antibodies simultaneously (Figure 3). If this format is chosen, it is important to pay close attention to the amount of antibody bound to the microspheres,

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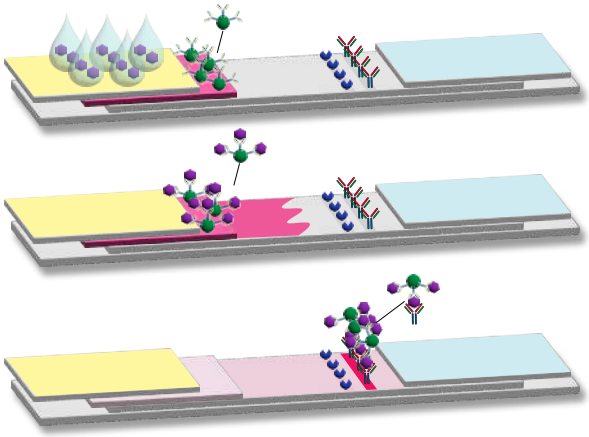
in relation to the amount of free antigen in the sample. If the sample does not contain an excess of free antigen, some of the microspheres will bind at the test line, giving a weak signal and making the test result ambiguous. In this type of test,

- Dyed beads or colloidal gold are conjugated to Antibody 1, which is specific for the biomarker antigen/analyte being tested. These are dried into the conjugate pad.
- The TL is coated with the antigen/analyte, which captures any beads or colloidal gold that was NOT bound by the antigen/analyte from the sample.
- The CL is coated with Antibody 2, which recognizes the Antibody 1.

A positive test result is when nothing appears in the TL, yet the CL is positive. A negative test result has a reaction at both the TL and CL.

A. Positive Test



B. Negative Test

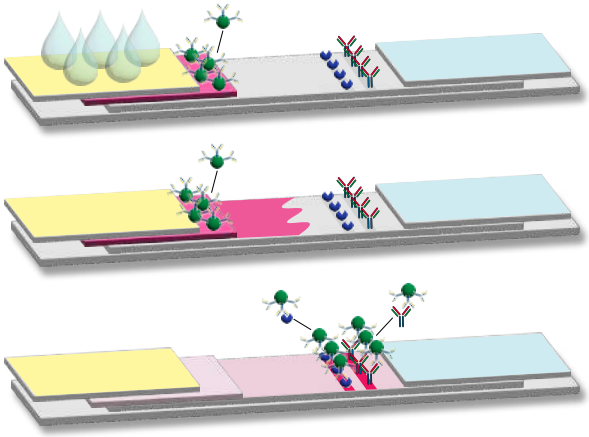
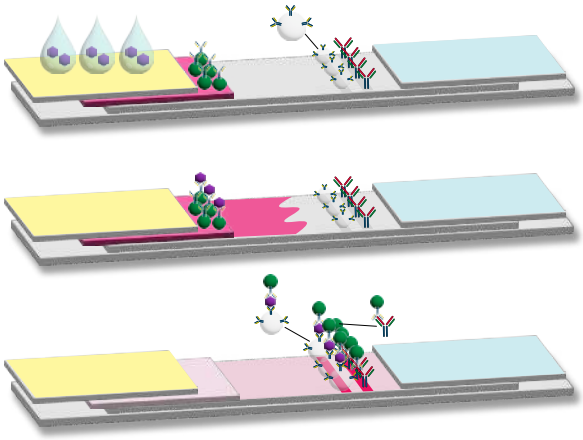


Figure 3 Competitive lateral flow assay (A) When antigens/analytes are found in the sample, if there is sufficient quantities available, the antibody:bead complexes become saturated. As they flow down the strip, saturated antibodies will not bind the test line (TL), but the antibody on the control line (CL) will bind. (B) In a negative result, the lack of antigen/analyte in the sample leaves unbound antibody1:bead complexes to flow through the membrane. These bind to the TL as well as the CL.

One variation to the above reaction schemes is the "Boulders in a Stream" approach (This gets around the problem of protein-coated microspheres sticking to the membrane nonspecifically by using a membrane that is inert and does not bind antibodies). This makes migration of the mobile phase antibodies very efficient and reliable. The capture antibodies, rather than being physically bound by the membrane, are attached to large microspheres, which will be held in place physically, rather than chemically, while the sample passes by, much like boulders in a stream. This can be used for both above-mentioned reaction schemes and is diagrammed in Figure 4.

A. Positive Test



B. Negative Test

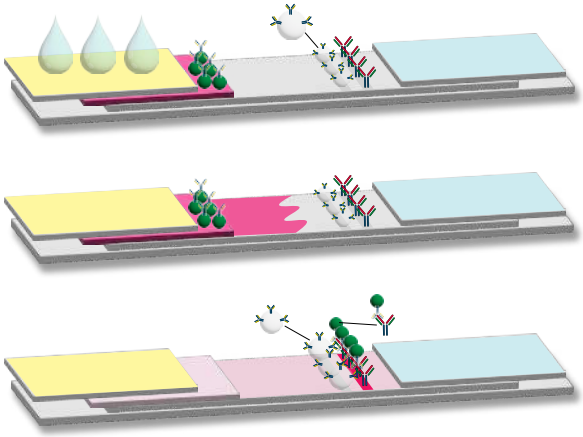


Figure 4 Boulder's in the stream lateral flow assay. (A) The positive test with antigen in the sample binds to antibody 1 in the sample pad. As it flows through the membrane, instead of antibody 2 being bound directly to the membrane it is bound to an unlabeled bead or "boulder". The antigen bound to antibody 1, also binds to antibody 2 and antibody 3 at the control line. (B) Negative results show an interaction of the bead at the control line, but not at the test line.

C. Quantitative Capabilities

By using the same format for lateral flow tests and dyeing the solid support with a visual dye, magnetic, or fluorescent dye, investigators have generated quantitative tests using LFT. If the spectral properties of the dyed microspheres to which the antibodies are conjugated are known, the amount of antibody bound at the capture line can be precisely quantified; this is the principle behind using a fluorometer. This not only presents opportunities for detection but provides avenues for developing existing lateral flow tests by converting them into quantitative assays.

IV. MATERIALS FOR LATERAL FLOW ASSAYS

The constituents of a lateral flow test can be shown in Figure 1 (see above). Each component plays a key role in the overall final performance of your

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assay. For a comprehensive review of the materials used in LFTs, please see (O'Farrell, 2009). The variable methods and materials can be complex, and that is why Ethos Biosciences has developed a cost-effective Lateral Flow Starter Kit that includes a selection of components for researchers developing a new LFT. LFA Starter Kit includes a wicking pad, assorted conjugate/sample pads, assorted nitrocellulose membranes, the backing card, and cassette for mixing and matching the different components to find the optimum combination for your specific sample, analyte/antigen, and antibody.

A. Membrane

The **membrane** (Figure 1, Part (1)) of the lateral flow test binds the designated proteins for both the TL and CL. Normally, the membrane is made up of primarily hydrophobic materials, such as nitrocellulose. Both the microspheres used as the solid phase supports and the antibodies are hydrophobic, and their interaction with the membrane allows them to be effectively dried onto the membrane. These hydrophobic interactions are reliable, so much so, that getting the hydrophobically bound antibody/microsphere complexes to enter the mobile phase upon sample introduction can be difficult. To circumvent this problem, the membrane should be blocked with proteins, surfactants, or polymers, so the pores of nitrocellulose are opened without trapping the microsphere complexes as the liquid flows from the sample pad to the wicking pad.

Key membrane parameters to consider when developing a lateral flow assay:

- Flow or wicking rate: The speed at which the sample liquid with beads or colloid gold move through the membrane must be slow enough for the binding to occur at the TL and CL.
- Protein binding capacity: The width of the TL and CL must be sufficient to bind enough proteins to have measurable results.
- Dispensing and drying the TL and CL: The method used to deposit proteins on nitrocellulose can affect the TL or CL protein's ability to interact with the beads as they flow through the region. The drying and storage methods also affect the TL and CL protein stability.
- Pore size: As the beads or colloidal gold complexes travel through the membrane, the pores must be large enough to allow passage at the correct rate. This parameter can be hard to determine since nitrocellulose is more like a sponge with irregularly sized and shaped gaps. The flow or wicking rate can be a better way to determine if the membrane suits your specific reagents.

B. Wicking pad

The **wicking pad** (Figure 1, Part (2)) should absorb the sample liquid containing excess microsphere complexes. The size of the wicking pad defines the maximum volume of sample needed for the assay since overloading the test with too much sample would cause back wash to the membrane reaction areas.

C. Conjugate pad

The **conjugate pad** (Figure 1, Part (3)) holds the dyed beads, fluorescent beads, or colloidal gold conjugated to the appropriate antibody. So, this is the area of the test where the analyte/antigen from the sample binds to the microspheres. The pad consists of glass fibers, polyesters, or rayons, which means there are some key parameters that must be optimized for each type of sample and the antigen/analyte that is being tested. These include:

- Stability: The pad must stably hold the microsphere complexes during storage without interfering the ability of them to interact with the analyte/antigen.
- Release rate: The pad must release the microsphere complexes correctly. Too fast of a release, and the analyte/antigen may not have sufficient time to react with the microspheres. Too slow of a release rate, the interaction with the membrane TL and CL regions within the defined time would be inefficient.
- Pretreatment options: As with the membrane, this pad may also need to be treated with proteins, surfactants, or polymers before adding the microspheres to facilitate the release rate. The addition of sucrose or trehalose can also increase the stability of the microspheres, dissolve easily when wet, and allow the flow of microspheres to be correct for the test.

D. Microsphere complexes

There are several sizes of types of polymers compatible with lateral flow tests (LFTs). The size of the microsphere should be 1/10th of the pore size in the membrane for efficient flow through the test strip. If using visible dyed beads, the following recommendations can facilitate clear visualization of the TL and CL:

- Whole blood: Black or dark blue
- Serum: Bright red or bright blue
- Urine: Green, blue, red, or black
- Saliva: Any dark color
- Cerebral spinal fluid: Any dark color

In addition to visibly dyed microspheres, these can be labeled with fluorescent dyes, fluorescent labels such as Europium chelate, magnetic/paramagnetic beads with or without dyes, etc.

Multiplexing Potential: Multiplexed LFTs can use the different colored beads to distinguish multiple analytes/antigens from one sample. Multiplexing can make field testing more efficient. By placing multiple lines of capture antibodies on the membrane, each for a different analyte, one can develop a single test for more than one analyte.

Attachment Chemistry: Adding the specific antibody to the microsphere can be done via:

- **Covalent Coupling**: Permanent attachment of the antibody to carboxyl groups on the surface of the microsphere. For more options and basic protocols, see Covalent Coupling.
- **Adsorption**: Relies on hydrophobic interactions between microsphere and protein. May result in random orientations for the antibodies, which could reduce the number of sites available to bind the analyte/antigen. For more information, see Adsorption to Microspheres.
- **Affinity Binding**: Use Protein A, Protein G, or secondary antibody coated microspheres that bind to the specific antibody. These particles bind many IgG's at the Fc region, allowing for optimized, directed antibody attachment. Microspheres are also available with streptavidin on the surface. If a specific orientation of the antibody is required, a biotin tag can be placed in the protein to facilitate the binding orientation on the bead. Additional information regarding these microspheres can be found in Affinity Coated Ligand microspheres.

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Products available: Bangs Labs has a full selection of beads for use in LFTs:

- Dyed Polystyrene Beads in vibrant red, blue, green, black, etc. and in different diameters. See Visibly Dyed Microspheres for more information.
  - Dyed Carboxyl Polystyrene Beads with carboxyl groups for covalent coupling of the antibody.
  - Dyed Protein-Coated Polystyrene with streptavidin attached for biotinylated proteins.
- Fluorescent Nanoparticles in polystyrene and magnetic options. We offer fluorescently labeled functionalized and non-functionalized polystyrene and fluorescently labeled carboxylated magnetic polymer beads in different diameters. Another LFT bead is our Europium Chelate Nanoparticles which have strong fluorescence emission and stability. See Europium Chelate Microspheres for more information. Bangs Labs offers an Europium Chelate COOH Sampler Pack to investigate which size particle is perfect for your specific test.
  - Incorporation of lanthanide fluorescent particles into an LFA can improve sensitivity and has been employed in recent device development (Chang *et al*., 2020). Europium Chelate particles are characterized by long Stokes shifts, or intervals between fluorescence excitation and emission maxima. Additionally, the fluorescence lifetime of lanthanide chelates (e.g., Europium chelate) is in the range of milliseconds, rather than nanoseconds (traditional fluorophores) like traditional fluorophores. These characteristics allows the particle signal to be easily distinguished from background autofluorescence, quantitatively due to the resolved fluorescence, which requires a time resolved fluorescent reader (TRF) instrument is required to read the results.
- Colloidal Gold offers high sensitivity and are visible without special instrumentation that is required for fluorescent nanoparticles. These are much smaller and are available in suspensions of OD1 and OD5. For more information see Colloidal Gold.

E. Sample pad

The **sample pad** (Figure 1, Part (4)) absorbs the actual sample and conditions it for the assay, which can include filtering impurities, adjusting buffer compositions such as pH, and establishing the initial flow rate. This region also mixes the sample with any additional surfactants, detergents, or blocking agents. The pad may also have additional agents to increase the reaction between the analyte/antigen with the antibody on the microspheres.

F. Membrane Backing and Plastic Housing

The **backing card** has an adhesive that holds each of the other components in the correct place. The distances between the components, how much they overlap, and the length of each of the components are important to the overall function. During assembly, the backing materials also provide the structure to ensure the materials do not move during the cutting process. The **plastic cassette** holds the final strip and provides openings for the sample to be added and to visualize the TL and CL results. The cassette is not used for LFTs that are designed as dipsticks.

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PRODUCT INFORMATION

COMPANY	LATERAL FLOW PRODUCTS
Bangs	Dyed Polystyrene
	Dyed Carboxyl Polystyrene
	Dyed Protein-Coated Polystyrene
	Europium Chelate PS - COOH
	Europium Chelate COOH Sampler Pack
	Gold Colloid
Ethos	LFA Starter Kit
	Gold Colloid

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