Guide to Lateral Flow Immunoassays

Innova Biosciences Guide

Lateral flow immunoassays
Components, process, assay formats and nanoparticles
Contents

1. Evolution of lateral flow immunoassays
2. Immunoassays
3. Lateral flow immunoassays
   3.1 The sample application pad
   3.2 The conjugate release pad
   3.3 The detection reagent
      3.3.1 The antibody
      3.3.2 The detection moiety
   3.4 The membrane
   3.5 The wicking pad
   3.6 The plastic cassette
4. Process options
5. Assay formats
6. Advantages and disadvantages of lateral flow immunoassays
7. Nanoparticles for lateral flow immunoassays
8. Custom services from Innova Biosciences
1. **Evolution of lateral flow immunoassays**

Lateral flow immunoassays are a well-established and extremely versatile technology that can be applied to a wide variety of diagnostic applications. Since their inception in the late 1980s a huge range of lateral flow immunoassays have been launched, with the global lateral flow immunoassay market expected to be worth approximately $6 billion by 2020. Lateral flow immunoassays are widely used in hospitals and clinical laboratories, as well as in veterinary medicine, in environmental assessment, and for safety testing during food production. Due to the low development costs and the relative ease of production, this list of applications continues to grow.

The technology on which lateral flow immunoassays are based was derived from the work of Singer and Plotz who, in 1956, developed a latex agglutination assay to diagnose rheumatoid arthritis. Latex particles were used as carriers for human antibodies; upon binding of these antibodies to rheumatoid factor from the serum of patients with rheumatoid arthritis the particles became cross-linked to form visible clumps in a process termed agglutination.

At around the same time as Singer and Plotz were developing their agglutination assay, the first radioimmunoassay, designed to measure insulin levels in human serum, was invented by Yalow and Berson. Guinea pigs were immunized with bovine insulin, to which they produced an immune response and generated antibodies. These guinea pig antibodies were incubated with human insulin and iodine131-labeled bovine insulin, both of which competed for antibody binding. The presence of insulin in human serum was subsequently detected by paper chromato-electrophoresis, and Rosalyn Yalow received a Nobel Prize for her work in the development of the radioimmunoassay.

Enzyme immunoassays evolved shortly after radioimmunoassays, providing a number of significant advantages including faster reaction times, greater specificities and, most importantly, the replacement of radioisotopes with enzymes. Other enabling technologies which progressed during this time included antibody production methodologies, nitrocellulose membrane manufacturing processes and liquid dispensing practices. All of these efforts culminated in the filing of three US patents in 1987 (from Becton Dickinson, Unilever and Carter Wallace) for the very first lateral flow immunoassays.

The main application to drive the development of these early lateral flow immunoassays was the human pregnancy test, which relied on the detection of human Chorionic Gonadotropin (hCG) in urine. hCG is a hormone which is secreted during the early stages of pregnancy, and plays an essential role in early pregnancy survival; its presence in urine can be used to determine a pregnancy. These first lateral flow immunoassays clearly demonstrated the utility of point-of-care (POC) testing, and paved the way for the evolution of a wide range of tests.

2. **Immonoassays**

An immunoassay is defined as a bioanalytical method which relies on the interaction between an antibody and an antigen (the analyte). A wide variety of immunoassay techniques has been developed, with Western blotting, immunocytochemistry (ICC), immunohistochemistry (IHC), flow cytometry and ELISA being amongst the most well-known. All of these methods share a number of advantages: they are highly selective, often have low limits of detection, are applicable to the determination of a range of analytes and are relatively inexpensive to perform.
The readout of an immunoassay can take a number of different forms. Colorimetric detection relies on the generation of a colored product, fluorometric detection requires the excitation and subsequent emission of light by a fluorophore, chemiluminescence occurs when a substrate is catalyzed by an enzyme and produces light as a by-product of the reaction, and another (less popular) option is the use of radiolabels. Since lateral flow immunoassays tend to be qualitative rather than quantitative, a colorimetric method is usually the preferred readout. The demand for quantitative lateral flow immunoassays is growing steadily, however the development of these presents a challenging goal since quantitative assays require considerably more from the finished product in terms of reproducibility, stability, sensitivity and dynamic range than is needed from a qualitative assay.

3. Lateral flow immunoassays

Lateral flow immunoassays, also known as immunochromatographic assays or strip tests, are immunoassays which have been designed to operate along a single axis. Although there are a number of different variations of the technology, they all operate using the same basic concept.

When a lateral flow immunoassay is run, the test sample is added to a sample application pad at the end of the strip. The sample then migrates to the conjugate release pad, where a detection particle (typically gold or latex) that has been conjugated to a biological component of the assay is held. Next the sample and the detection reagent migrate to the reaction membrane; a second biological component of the assay will have been immobilized here to function as a capture reagent. The capture reagent usually exists as a test line which spans the width of the membrane; a control reagent will be immobilized in a second line further along the membrane. The analyte is either captured at the test line, or continues to migrate until reaching the absorbent wicking pad at the other end of the strip. The detection reagent binds at the control line to indicate that the assay has run successfully. This process is illustrated in figure 1.

Figure 1. Schematic representation of a typical lateral flow assay. The antigen is illustrated in yellow.
3.1 The sample application pad

This is an absorbent pad on to which the sample is applied, and is typically composed of a woven mesh or cellulose fiber. Woven meshes have good tensile strength and a low bed volume, meaning that they retain very little sample volume; they can though be rather expensive. Cellulose fiber pads have low tensile strength and a much greater bed volume; they are also relatively cheap. Irrespective of which material is chosen, the sample application pad should exhibit consistent absorbency, thickness and density so that uniform wicking rates ensure assay reproducibility. The sample application pad should also demonstrate low protein binding to avoid loss of analyte.

The main function of the sample application pad is to promote even and controlled distribution of the sample, however the sample application pad can be modified to enable conditioning of the sample. By pre-treating the sample application pad with components such as proteins, detergents or salts it is possible to reduce non-specific binding, increase the sample viscosity, or alter the pH. Sample conditioning is an important consideration due to the very different nature of the samples which may be tested in a lateral flow immunoassay; these can be as diverse as water, urine, serum, plasma, blood, saliva, cerebrospinal fluid, milk, amplified nucleic acids, and solubilized solid materials such as feces, food, plants and soil. Due to the low bed volume it is impractical to pre-treat woven meshes; cellulose fibers are much more amenable to these modifications.

3.2 The conjugate release pad

The conjugate release pad is typically composed of non-woven glass fiber, into which the detection reagent has been dried. Once the sample application pad has been saturated, the sample flows in to the conjugate release pad where it releases the detection reagent; the detection reagent then leaves the conjugate release pad and moves with the sample in to the membrane.

The role of the conjugate release pad is to ensure uniform transfer of the sample and the detection reagent, and to preserve the conjugate upon drying and re-wetting. It plays a pivotal role in controlling the performance of the lateral flow immunoassay. The conjugate release pad should exhibit low non-specific binding so that the detection reagent does not remain trapped in the pad. A pre-treatment may be necessary to increase wettability, decrease non-specific interactions and to control the pH. It is important that the conjugate release pad has a consistent bed volume to ensure that the amount of detection reagent in each test strip remains constant.

3.3 The detection reagent

The detection reagent employed in a lateral flow immunoassay typically consists of antibodies which have been conjugated to colored or fluorescent particles. The overall effectiveness of the finished lateral flow immunoassay will be dependent on the quality of both the antibodies and the detection moiety, amongst other factors.
3.3.1 The antibody

When selecting suitable antibodies for use in a lateral flow immunoassay there are a number of factors which must be taken into consideration. Firstly, it is critical to ensure a consistent supply of good quality antibodies. Secondly, the antigen specificity should be assessed. The antigen specificity is defined by the antibody’s variable region; if this region recognizes a common molecular motif, the antibody may bind to the same epitope on multiple targets. Since non-specific binding could lead to the misinterpretation of results it is preferable to select an antibody which recognizes a single target.

The binding affinity of the antibody is also important; unless the lateral flow immunoassay is intended for the detection of an analyte which is present at relatively high concentrations in the sample, an antibody with high binding affinity is required. The binding affinity is defined by the equilibrium dissociation constant, $K_D$, and is governed by the same principles which define any reversible biomolecular interaction:

$$\text{Ab} + \text{Ag} \rightleftharpoons \text{Ab-Ag} \quad K_D = \frac{[\text{Ab}][\text{Ag}]}{[\text{Ab-Ag}]}$$

In this equation, $[\text{Ab}]$ is the molar concentration of unbound antibody, $[\text{Ag}]$ is the molar concentration of unbound antigen, and $[\text{Ab-Ag}]$ is the molar concentration of the Ab-Ag complex. The lower the $K_D$ value, the higher the affinity of the antibody for its target, and therefore the less antibody that is required in the lateral flow immunoassay for detection of the analyte. Antibodies with high binding affinity typically have a $K_D \leq 10^{-7}$M.

As well as being desirable to use an antibody with high binding affinity, in a lateral flow immunoassay format it is vital that the forward reaction is driven by a fast on-rate. The on-rate defines how quickly the antibody binds to the antigen to form a complex. This is of particular importance for the reaction which occurs at the test line, since the time which the analyte spends in contact with the capture antibody that has been immobilized here will be just a few seconds. Although the flow rate and the width of the test line can both be manipulated to affect this contact time, a fast on-rate is essential to a robust lateral flow immunoassay. A fast on-rate is also important during the binding of the analyte to the detection antibody, although the analyte will spend slightly longer in the presence of the detection antibody since the detection antibody is located nearer to the beginning of the test strip. A common approach to finding suitable antibody pairs is to perform an ELISA, however the assay conditions of an ELISA are very different to those of a lateral flow immunoassay, with incubation times in the region of hours rather than seconds. Because of this, it is important to test the antibodies as soon as possible in a format that can predict their performance in a lateral flow immunoassay.

Another consideration when selecting antibodies for a lateral flow immunoassay is whether to choose monoclonals or polyclonals. Monoclonals are preferred since large quantities of a specific antibody, recognizing a single antigenic epitope, can be produced. Although polyclonals can produce adequate results, they are subject to variability between animals and over time, and a supply cannot be guaranteed for the lifetime of the lateral flow immunoassay.
Whether a monoclonal or a polyclonal antibody is selected, it should minimally be affinity purified since any contaminating proteins may compete for binding during the assay. The antibody must also have sufficient stability to withstand the various extremes that it will be subjected to during manufacture and storage of the lateral flow immunoassay device; it needs to be able to maintain its reactivity after being adsorbed to the conjugate release pad, it should tolerate being completely dried for a defined time period (the expiration date of the lateral flow immunoassay), and it should instantly be reactive following rehydration by the sample.

### 3.3.2 The detection moiety

The most commonly used detection moieties in lateral flow immunoassays are gold nanoparticles or latex beads. These particles produce a colored readout which requires no development process for visualization. Fluorescent labels, enzymes, other colloidal metals and magnetic particles can also be employed, and interest in these is growing steadily.

When working with gold nanoparticles or latex beads, it is essential that these particles are of a uniform size and have a regular spherical shape. This ensures a consistent rate of transfer through the membrane; small particles will move more rapidly than large particles, so any discrepancies in size can manifest as different apparent sensitivities. The particles should also demonstrate reproducible conjugation to the antibody, the conjugation procedure should be easy and scalable, and the particles should be reasonably priced.

Traditionally, passive adsorption methods have been used to attach antibodies to detection moieties however passive adsorption can be time-consuming and requires specialist knowledge. Covalent conjugation allows the production of highly stable conjugates, and uses considerably less antibody than passive adsorption methods. The direct conjugation of antibodies or proteins to gold nanoparticles or latex microspheres can be carried out quickly and easily using InnovaCoat® GOLD and LATEX conjugation kits from Innova Biosciences.

### 3.4 The membrane

The membrane is considered to be the most important element in a lateral flow immunoassay. Nitrocellulose is the most commonly-used material, although cellulose acetate, polyvinylidene fluoride (PVDF), charge-modified nylon and polyethersulfone (PES) may also be employed. Nitrocellulose membranes exhibiting a range of pore sizes (0.05 to 12μm) are commercially available, but since the pores do not have an even distribution it is more appropriate to consider capillary flow time when selecting a membrane for the lateral flow immunoassay. Capillary flow time is typically expressed as seconds/cm and is defined as the time taken for the sample liquid to travel to and completely fill the strip of membrane. The capillary flow time can affect the sensitivity and specificity of the lateral flow immunoassay, as well as the consistency of the test line.

Capture antibodies are immobilized across the membrane, typically in two lines. The test line is used to bind the sample protein, while the control line consists of species-specific antibodies for the detection antibodies (e.g. anti-mouse antibodies) and is used to demonstrate that the lateral flow immunoassay is performing as it should be. Instrumentation is required for precise application of the capture antibodies;
positive displacement systems and air jetting systems are most commonly used. In positive displacement systems a liquid stream is dispensed directly on to the membrane, either using non-contact or contact tips. Non-contact tips are suspended at a fixed distance above the membrane, while contact dispensing requires a flexible tube to be dragged across the surface of the membrane at the same time as the liquid is dispensed. In both cases it is critical that the membrane is of a consistent thickness to avoid causing any mechanical damage. In air-jetting systems the liquid stream is injected with pressurized air to form an aerosol and is then dispensed on to the membrane. Once again a constant membrane thickness is vital, since dispersion of the aerosol will be greatly affected by the distance between the tip and the membrane.

To avoid assay variability it is imperative that the membrane remains perfectly flat while it is being striped with antibodies, and for this reason it may be preferable to select a nitrocellulose membrane with a non-porous backing film. These are readily available, and are much easier to handle than unbacked membranes. If the test strip is to be placed in a plastic housing it is important to ensure that the thickness of the plastic backing is consistent otherwise the test strip can be subjected to variable levels of compression.

In addition to the choice of membrane, a number of other variables should be taken in to consideration when striping the membrane. These include the concentration of the capture antibodies, the choice of antibody diluent, the reagent dispensing rate and the drying method. Complete drying is critical to ensure that the capture antibodies become fixed to the membrane, however it is important to note that backed membranes take considerably longer to dry than unbacked membranes.

3.5 The wicking pad

This is found at the end of the lateral flow immunoassay, and functions to increase the volume of sample which enters the test strip. The increased volume can be used to wash away unbound detection antibodies thereby reducing background and increasing assay sensitivity. Wicking pads also prevent backflow. The wicking pad is typically composed of a cellulose filter; by changing the dimensions of the wicking pad it is possible to optimize the total volume that is taken up by the test strip.

3.6 The plastic cassette

For ease of use, many lateral flow immunoassays are placed in to a plastic cassette. The aim of this is primarily to ensure that the end user applies the sample only to the sample application pad, and not to any other region of the test strip. The plastic housing also protects the test strip. During the manufacturing process the plastic housing can be labeled, for example to clearly indicate the position of the test line and the control line. Plastic cassettes are available as off-the-shelf products, and the choice of cassette for the finished test should be matched to the dimensions of the test strip.

4. Process options

There are two general approaches to lateral flow immunoassay assembly, namely batch processing and in-line or reel-to-reel assembly. The batch processing approach allows for the use of low-cost equipment
and is suitable for relatively low production volumes, however its disadvantages are that it involves a high degree of manual labor and can be prone to product variability. During in-line manufacturing, lateral flow immunoassay strips are machine-processed in continuous rolls; this results in higher throughput and considerably lower product variability.

Whether carrying out batch processing or in-line assembly, it is vital to control the ambient conditions (temperature and relative humidity) during pad drying, dispensing processes and strip assembly. Protein dispensing is usually performed at 18-25°C with 40-60% relative humidity, while strip assembly and storage is generally carried out at 18-25°C with <20% humidity. Fluctuations in these conditions can introduce an additional source of variability to the lateral flow immunoassay.

5. Assay formats

The two main approaches which are used in a lateral flow immunoassay are a direct (sandwich) assay format and a competitive assay.

In a sandwich assay the analyte is captured between two complementary antibodies. One of these antibodies is conjugated to the detection reagent and is held at the conjugate release pad, while the other antibody is immobilized at the test line on the membrane. Any excess labeled antibody will be captured at the control line. The intensity of the color which is seen at the test line can be indicative of the amount of analyte present in the sample. This type of test is generally used for larger analytes with multiple antigenic sites.

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Figure 2. Sandwich assay format. A positive result is seen when the analyte becomes bound to the detection antibody and to the capture antibody which has been immobilized at the test line. The antibody which has been immobilized at the control line will recognize the detection antibody, producing a colored line to indicate that the lateral flow assay has performed as expected.
The complexity of the lateral flow immunoassay can be increased through the use of multiple test lines, allowing the detection of multiple antigens within the same sample. A recent trend is to use dots instead of lines for multiplexing analysis. Dots can also be used to produce alpha-numeric symbols on the membrane, allowing intuitive result generation.

Competitive assays are used for smaller analytes which have a single antigenic determinant and therefore cannot bind two antibodies simultaneously. In this type of assay format the antigen is typically immobilized at the test line. If the analyte is present in the sample, it binds to the detection antibody which is subsequently unable to bind at the test line. A lack of signal at the test line is therefore indicative of a positive result.

Figure 3. Competitive assay format. A positive result is seen when the analyte becomes bound to the detection antibody and therefore cannot bind to the antigen which has been immobilized at the test line. The antibody which has been immobilized at the control line will recognize the detection antibody, producing a colored line to indicate that the lateral flow assay has performed as expected.

Although many different permutations are possible, all lateral flow immunoassays involve the formation of a complex between a detection reagent that is free in the sample stream, and a capture reagent which is bound to the membrane at the test line.

6. **Advantages and disadvantages of lateral flow immunoassays**

The advantages of the lateral flow immunoassay system are well-known. Most important among these are the point-of-care (POC) nature of the technology, the broad range of applications, and the speed and relatively minimal cost at which a newly-developed lateral flow immunoassay can be brought to market.
The disadvantages are less obvious, however sensitivity and reproducibility are the most challenging obstacles which must be overcome when developing a lateral flow immunoassay.

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Low cost</td>
<td>Qualitative or semi-quantitative readout</td>
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<tr>
<td>Wide range of applications</td>
<td>Restriction on total test volume can impose a limit on sensitivity</td>
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<tr>
<td>Well-established technology, ease of manufacture</td>
<td>Inaccurate sample volume may reduce precision</td>
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<tr>
<td>Long shelf-life, no need for refrigeration</td>
<td>Test-to-test reproducibility may be problematic</td>
</tr>
<tr>
<td>Simple, user-friendly operation</td>
<td>Difficult to miniaturize sample volume</td>
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<tr>
<td>High sensitivity and specificity</td>
<td>Multiplexing can be challenging</td>
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<tr>
<td>Low sample volume required</td>
<td>Unclear patent situation in some instances</td>
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<tr>
<td>One-step assay, no washing steps necessary, short time to result</td>
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<tr>
<td>Relatively short timeline for development, time to market is reduced</td>
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<tr>
<td>High potential for commercialization</td>
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<tr>
<td>Easily scalable</td>
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<tr>
<td>Can be integrated with reader systems</td>
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<tr>
<td>Possibility of multiplexing</td>
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Figure 4. Advantages and disadvantages of lateral flow immunoassays.

7. Nanoparticles for lateral flow immunoassays

Gold nanoparticles and latex beads are the most widely used labels in commercial lateral flow immunoassays. Gold nanoparticles are well-suited to this purpose because of their intense ruby red color, and Innova Biosciences offers colloidal gold nanoparticles that are ideal for lateral flow immunoassays. Our colloidal gold has been developed using specialized techniques which enable the production of extremely uniform spherical particles with a narrow size distribution, thereby minimizing assay variability. They can be produced in large quantities without compromising on quality. Figure 5 shows a TEM image of our 40nm colloidal gold.

Figure 5. TEM image of 40nm colloidal gold, demonstrating the uniform spherical shape and narrow size distribution.
The benefits of Innova Biosciences’ colloidal gold include:

- Different nanoparticle sizes and concentrations available
- Fully scalable - available at high concentration (>20 OD) and large volume (liters)
- Stringently QC tested
- Uniform spherical shape and narrow size distribution
- Competitively priced

The use of colloidal gold for lateral flow immunoassays requires passive adsorption of the antibody onto the surface of the nanoparticle, a process which can be extremely challenging and necessitates specialist knowledge. To overcome this, we have developed InnovaCoat® GOLD conjugation kits. These contain ‘conjugation friendly’ nanoparticles that have a proprietary surface coating which greatly enhances gold stability and permits easy covalent attachment of a variety of molecules, including antibodies, analytes and other biomolecules. InnovaCoat® GOLD conjugates can be used in a range of assay formats, including lateral flow immunoassays, and demonstrate enhanced sensitivity when compared to traditional passive conjugation methods, as illustrated in figure 6.

![Figure 6](image-url)

Figure 6. Competitive lateral flow immunoassay comparing antibodies conjugated to 40nm gold particles by a traditional passive method against those conjugated covalently using InnovaCoat® GOLD.
The benefits of InnovaCoat® GOLD include:

- Quick and easy to use
- Proprietary surface coating prevents metal-protein interactions
- Different nanoparticle sizes and amounts available
- Freeze-dried
- Fully scalable
- Stringently QC tested: uniform spherical shape and narrow size distribution
- Covalent conjugation uses significantly less antibody than passive conjugation and provides a highly stable conjugate

Latex beads are another popular choice for lateral flow immunoassays since they are available in a range of vibrant colors, making them easily visible and allowing for the simultaneous detection of different analytes.

![Multiplexed lateral flow immunoassay using Innova Biosciences' latex beads. Latex beads of three colors have each been conjugated to a different antibody or protein.](image)

Figure 7. Multiplexed lateral flow immunoassay using Innova Biosciences’ latex beads. Latex beads of three colors have each been conjugated to a different antibody or protein.

For use in a lateral flow immunoassay, latex beads require conjugation to the detection antibody. Traditional methods of latex conjugation are technically complex and can waste a lot of precious material; passive conjugation requires extensive optimization to identify the appropriate pH, while covalent conjugation is prone to aggregation of the latex. Innova Biosciences’ latex conjugation kits have been designed to greatly simplify the process. These kits allow antibody conjugation to blue, red or black latex beads without the need for extensive optimization. High yields of functional conjugates can be made without the need for harsh resuspension methods such as sonication and vortexing.
The benefits of Innova Biosciences’ latex conjugation kits include:

- Quick and easy to use
- Different colors available
- Freeze dried
- Fully scalable
- Stringently QC tested
- No extensive pH optimization required
- Resistant to aggregation
- Covalent conjugation provides a highly stable conjugate

For further information on our full range of colloidal gold, InnovaCoat® GOLD and latex products, please see our website.

8. Custom services from Innova Biosciences

Although lateral flow assay development can be relatively straightforward, we recognize that sometimes you might need some assistance. You may, for example, be struggling to optimize your antibody conjugation, time restraints could mean that you wish to outsource your bulk conjugations, or you might be a relative newcomer to lateral flow. To help you streamline your workload, Innova Biosciences offers a wide range of custom services, including our lateral flow assay development service.

Our lateral flow assay development service is designed to take your lateral flow immunoassay from an initial idea, through to R&D, and then on to trusted partners for bulk manufacturing. The full service is a multi-step project providing you with different options, from a proof-of-principle lateral flow immunoassay using a dipstick, to full strips with sample application pad, conjugate release pad and wicking pad, and small scale manufacture.

Module 1 – conjugation services

Module 1 consists of our conjugate micro-optimization services, and our custom conjugate formulation services. Our conjugate micro-optimization services enable you to find the best performing conjugate for your assay, using very small quantities of your antibody or protein; our experienced conjugation scientists will produce a range of different antibody-gold nanoparticle conjugates for you to test in your application. Our custom conjugate formulation service can be used as a follow-up service from our conjugate micro-optimization service, or as an independent option; our team of expert conjugation scientists will quickly provide you with bulk quantities of your bespoke antibody-gold nanoparticle conjugate, in your chosen buffer and at your preferred concentration.
Module 2 – assay development

Our lateral flow assay development service is provided as a dipstick assay format, or as a full lateral flow test strip. The dipstick format consists of a wicking pad and a nitrocellulose membrane, and is designed to prove that the conjugation has been successful. The antibody-gold nanoparticle conjugate is mixed with the sample containing the antigen, and is then applied to the dipstick; a capture antibody on the nitrocellulose membrane is used to show that the assay set up is correct. The full lateral flow test strip format provides the same materials as the dipstick assay, with the addition of a conjugate release pad containing the dried antibody-gold nanoparticle conjugate, and a sample application pad.

Whichever format is chosen, the capture antibody is dispensed on the nitrocellulose membrane as either a spot or as a test line; greater amounts of antibody are required for the latter to allow for priming of the dispenser. If spots are dispensed on the nitrocellulose we will provide images (qualitative method), whereas if lines are dispensed the signal will be read and the sensitivity calculated (quantitative method).

Module 3 – lateral flow assay optimization

Within module 3, our experienced in-house team will determine the optimal amount of capture antibody and will test different pads and membrane treatments to provide you with your optimized lateral flow assay. Assay optimization will provide you with the best possible performance at the lowest manufacturing cost.

Module 4 – strip manufacturing for LFA

This service allows you to purchase assembled strips for your lateral flow assay, either as dipsticks or as full lateral flow test strips.

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