Lateral Flow Assay Development Guide

This document provides instructions to help maximize the sensitivity and performance of your lateral flow assay. We provide a step-by-step walkthrough of all of the stages of lateral flow assay design, provide best-mode protocols including specifics on material selection, and provide guidance on optimization strategies to further increase specificity and sensitivity. Successful lateral flow devices are the product of many small optimizations that are different with each particle type, target and system. We hope that you find this information useful and if you have any questions or comments, please feel free to contact us at info@nanocomposix.com or (858) 565-4227 x 2.

Contents

Introduction to Lateral Flow

Overview

The increasing prevalence of infectious diseases has heightened demand for easy-to-use, rapid in-vitro diagnostics (IVDs) and driven an explosion in public adoption of the Lateral Flow Immunoassay (LFA) format in recent years. LFAs are scalable, don't require instrumentation to interpret, are inexpensive to manufacture, require no formal training to use, and can provide users with accurate results at home or in a lab – a set of benefits few technologies can tout in the IVD space.

With the value of LFA highlighted by the SARS-CoV-2 pandemic, there has been a marked expansion in the use LFAs in new and innovative markets such as cancer screening, GMO testing, and veterinary medicine. Further, the development and incorporation of next-generation reporter particles, like gold nanoshells, has helped to produce more sensitive antibody-probe conjugates and aided the expansion of LFA to more challenging sample types such as bioaerosols and saliva, which provide increasing advantages in accessibility and ease-of-use.

Figure 1: A lateral flow assay utilizing high sensitivity gold nanoshell reporter particles that produce a blue-green line.

Contract development and manufacturing (CDMO) houses play a vital role in helping diagnostic developers and visionaries bring high-impact LFA products to market. For clients with experience developing LFAs, as well as those with limited exposure to the platform, a CDMO will function as a guide through the complexities of product development while helping to lower the burden of development by providing niche expertise without the infrastructure and hiring investment. The selection of a CDMO at any phase of development (e.g., feasibility, product development, and transfer to manufacturing) is critical to the future outcome of an LFA product and each house will have a unique approach to addressing technical challenges and client collaboration. At Fortis Life Sciences, we are proud to offer end-to-end lateral flow service solutions, tailoring each project to the unique demands of the assay. Critical to the Fortis Life Science approach to being a CDMO is the emphasis on transparency and education. The following guide serves as a demonstration of our approach to lateral flow assay development and aids in the understanding and optimization of lateral flow assays.

Commercially available COVID antigen test
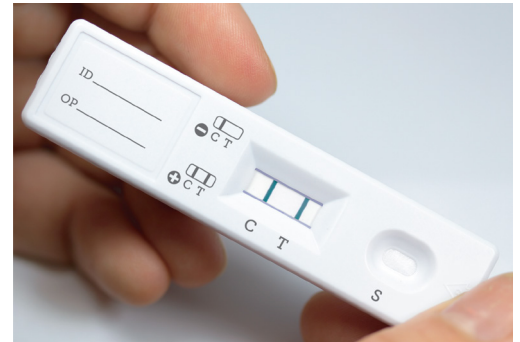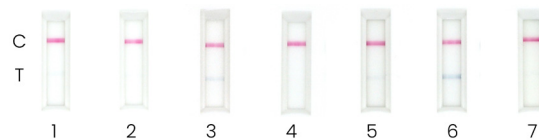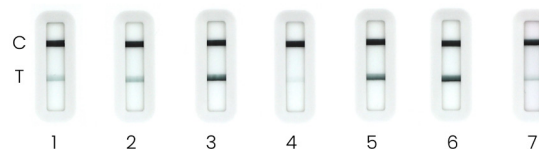
COVID antigen test with gold nanoshells

Figure 2: 7 positive samples run on a commercially available COVID antigen test using cellulose nanobeads vs. a COMD antigen test using gold nanoshells developed by nanoComposix

Lateral Flow Components

The lateral flow assay contains several key components:

- Sample pad: Traditionally the sample pad is the first material to come into contact with the sample, and functions to process the sample while controlling the flow of the sample onto the conjugate pad.

- Conjugate pad: A medium for dispensing and drying nanoparticle-antibody conjugates, aids in the controlled release of re-solubilized conjugate onto the nitrocellulose membrane

- Nitrocellulose membrane: Provides the ideal solid phase for immobilizing test and control line reagents

- Wick/absorbent pad: Provides uniform capillary flow through the membrane, absorbs applied sample, and prevents backflow
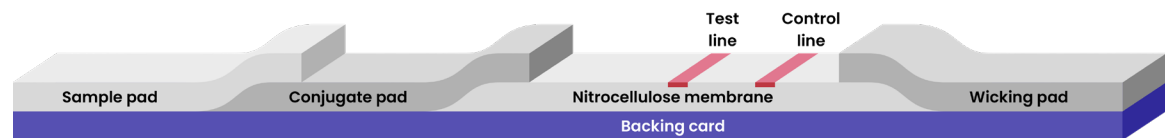

Figure 3: Schematic of a generic lateral flow test. The **sample pad** absorbs the sample and transports the sample to the conjugate pad. The **conjugate pad** contains the dried down antibody-nanoparticle conjugate. The nitrocellulose membrane has test and control lines that show the assay results. The **wicking pad** continues to pull the sample through the strip at an even rate. All components are assembled on a **backing card**.

A traditional lateral flow assay contains a sample pad, conjugate pad, nitrocellulose membrane, and a wick/absorbent pad that are all applied to an adhesive backing card (**Figure 3**). The controlled overlap between all these components allows the sample to move through the test strip via capillary action. The strip components are often housed inside of a plastic cassette so that only the sample pad and nitrocellulose membrane with the test and control lines are visible to the end user. A few drops of fluid are applied to the sample pad and the presence or absence of a test line after a short amount of time (12-20 minutes) indicates the presence or absence of the target analyte.

At the core of a lateral flow assay are the "conjugates" which are also called detector nanoparticles or probes. These brightly colored nanoparticles are functionalized with proteins, most often antibodies, that recognize the target analyte (e.g. the hormone hCG in the case of a pregnancy test). The readout of the assay occurs on a nitrocellulose strip that has two lines striped on the surface: a test line and a control line (Note: Multiple test lines specific to various targets can be included on a single strip. This is commonly referred to as a multiplexed assay). The test line contains an immobilized protein that either binds to the target analyte or competes with the target analyte for binding to show a positive or negative result. The control line contains an immobilized antibody that binds to the antibody on the surface of the particle whether or not the analyte is present, to confirm that the assay is working correctly. The control line is typically a secondary antibody specific to the host species of the primary antibody on the conjugate. The sample to be analyzed (e.g., blood, serum, plasma, urine, saliva, or solubilized solids) is added to the sample pad and is drawn through the lateral flow device by capillary action. The sample pad can filter unwanted portions of the sample (such as red blood cells or solid particulates) and normalize the pH of the sample if needed before the sample reaches the conjugate. The objective is to 'normalize' each sample to overcome sample-to-sample variability and ensure accurate and reproducible results each time the test is run. The liquid then wicks to the conjugate pad which contains the dried nanoparticle conjugate. The nanoparticle conjugate is solubilized on contact with the aqueous sample and can bind to the analyte of interest (if present). The nanoparticles and sample continue to flow through the nitrocellulose membrane until they reach the test line and control line. Binding events at the test line provide a visual indication of whether or not the analyte was present in high enough quantities to be detected.

Assay Formats

Lateral flow assays (LFAs) are an extremely flexible platform that can detect a wide range of targets and can be configured in a variety of orientations. One of the first steps in the design of a lateral flow assay is to understand which LFA format is right for the target analyte. Two common formats, "sandwich" and "competitive" assays, are described below and shown in **Figure 4**.

Sandwich Format: The sandwich assay format is typically used for detecting relatively large analytes. If the analyte has at least two distinct binding sites (i.e., epitopes), a "sandwich" assay can be developed in which an antibody to one epitope is conjugated to the nanoparticle and an antibody to another epitope is immobilized at the test line. If the analyte is present in the sample, the analyte will become the "meat" of the sandwich binding the nanoparticle conjugate to the test line, yielding a positive signal. The sandwich format results in a signal intensity that is proportional to the amount of analyte present in the sample.

Competitive Format: A competitive format is used for detecting analytes in which the analyte is too small for two antibodies to bind simultaneously, such as hormones and drugs of abuse. In a competitive assay, the test line may contain the target analyte molecule (usually a protein-analyte complex to help with stability on the nitrocellulose membrane). The nanoparticles are conjugated to an antibody specific to the target analyte. If the analyte is not present in the sample, the nanoparticle antibody conjugates will bind to the analyte at the test line, resulting in a high signal intensity. If the target analyte is present in the sample, the analyte will bind to the antibodies on the nanoparticle surface and prevent the nanoparticle from binding to the test line. This will reduce the signal at the test line resulting in a signal intensity that is inversely proportional to the amount of analyte present in the sample.
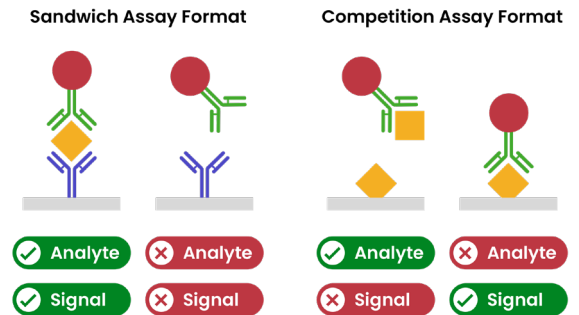

Figure 4: Schematic of sandwich assay format and competitive assay format.

To verify that the assay has been manufactuered and run correctly and indicates a valid result, both assay formats typically utilize a "control" line immobilized on the nitrocellulose membrane located after the test line. The control line is typically a species-specific secondary antibody that will bind to the antibody that is conjugated to the nanoparticle probe. For both assay formats the control line should be visible whether or not the analyte is present in the sample. Commonly, if a control line does not appear, it is indicative that there was a problem with the test and the result is not valid.

For many lateral flow diagnostics, a binary result interpretation (i.e., "yes" or "no") is the preferred endpoint (e.g., antigen test, pregnancy test, etc.). For other lateral flow assays, the intensity of the test line can provide "semi-quantitative" results, where the result is reported as falling within a particular range (e.g., low, medium, or high) or "quantitative" results, where a number that correlates to the concentration of the analyte is reported. Quantitative lateral flow assays require more stringent fabrication conditions and a digital reader. Advancements in the development of inexpensive readers either on-strip or based on cell phone technologies have recently become available and provide a simple and inexpensive solution for quantifying lateral flow assay results (**Figure 5**).

Figure 5: (A) Lumos Diagnostics on-strip reader. (B) Lumos Diagnostics semi-disposable reader with Bluetooth connectivity capabilities. (C) Novarum hardware-free smartphone reader

Nanoparticles

Fortis has extensive expertise in the synthesis, characterization, and surface modification of nanoparticles. We have been making highly engineered inorganic particles for more than fifteen years and have developed particles specifically engineered for both their optical properties and the conjugation to affinity ligands such as antibodies.

The methods and techniques used to conjugate antibodies to the surface of nanoparticles are critical for optimizing the performance of lateral flow assays. When using gold nanoparticles, antibodies can either be physisorbed to the surface, also referred to as passive adsorption/conjugation, or they can be covalently attached. In both passive and covalent coupling reactions, the purity, affinity, and cross-reactivity of an antibody or other ligand are important for developing sensitive and specific tests. It is important to purify and transfer all antibodies to the appropriate buffer before use in a conjugation reaction.

It is important to note that the guidance provided here is specific to conjugation procedures for binding antibodies to gold nanoparticles. While antibodies are the most common affinity ligand used in lateral flow tests, other molecules can also be attached to nanoparticles such as small peptides and other proteins (BSA, streptavidin, etc.).

Passive Adsorption: Passive adsorption is the traditional method for attachment of proteins to lateral flow nanoparticle probes and is still widely used. The mechanism of passive adsorption is based on van der Waals and other weak attractions between the macromolecule (such as antibody or protein) and the surface of the particle. The resulting forces between the antibody and the nanoparticle probe are influenced both by the nanoparticle surface and by the coupling environment. In the case of less hydrophobic antibodies or a more hydrophilic surface (i.e. −COOH modified), attachment by both ionic interactions and hydrophobic interactions can occur. Small changes in pH can alter the association dynamics and affect the efficiency of conjugation. A pH titration and an evaluation of the antibody-to-particle ratio needs to be performed to identify conditions where antibody adsorption is optimal. It is recommended that the pH of the adsorption buffer is slightly above the isoelectric point of the protein, which varies from antibody to antibody. The Fc portion of the antibody is generally more hydrophobic and therefore more likely to be adsorbed as compared to the Fab portion, offering some control over binding orientation. A large excess of antibody with respect to nanoparticle surface area is typically used to ensure dense surface binding and high salt stability of the gold post conjugation. There are two major drawbacks to passive adsorption. Firstly, every antibody requires slightly different conditions which require extensive optimization. Secondly, some antibodies may detach from the nanoparticle surface under certain storage conditions which can lead to a decrease in sensitivity and variability in results.

Covalent Coupling: Increasingly, LFA developers are covalently binding antibodies to the surface of nanoparticle probes. Covalent attachment is more stable with less antibody desorption and requires fewer antibodies during conjugation. Covalent attachment can be accomplished with several different types of chemistry. For our BioReady products that are optimized for lateral flow, we normally rely on amide bond formation to connect a carboxylic acid functionalized nanoparticle to free amines on the antibody. This covalent bond is achieved through an EDC/Sulfo-NHS intermediary generated from a carboxylic acid surfaced particle (Figure 6). For antibodies, lysine residues are the primary target sites for EDC/NHS conjugation. A typical IgG antibody will have 80 − 100 lysine residues of which 30−40 will be accessible for EDC/NHS binding. Proteins such as bovine serum albumin have similar numbers of surface-accessible lysine groups. Fortis sells BioReady nanoparticles with carboxylic acid surfaces, as well as an NHS-activated surface to allow for simplified conjugation that eliminates the need for the user to perform the intermediary EDC/NHS chemistry steps. In addition to its use in lateral flow, the same particle surface chemistry can be used to bind many other amine containing targeting ligands to the particle surface.

Preliminary Considerations

Before starting the development process of a lateral flow assay there are many aspects to consider. Based on the target analyte and the intended use, determine whether your assay will be competitive or sandwich and whether it will be qualitative, semi-quantitative, or quantitative. Other factors such as the sample type that will be used for analysis, the time to result, the desired sensitivity, and the required dynamic range will form the basis of the product specification and guide future work. Most of these decisions are driven by clinical relevance. It is important to consider all design inputs that are required before starting assay development. A critical first step is to identify a control assay, whether in lateral flow or on a different platform, that can independently measure the analyte of interest and validate the assay if this is intended for clearance through regulatory agencies such as the FDA. Next, the capture and detector antibodies for conjugation need to be identified and sourced. Depending on the application, antibodies may or may not be available commercially, and the number of clones and types of antibodies may vary considerably. How the antigen will be obtained and screened for development and optimization of the assay is also important. Ideally, the same antigen that was used to develop the commercial antibodies will be available and if possible, this antigen should be available from multiple sources to identify variances. Aspects to consider in antigen selection include the storage buffer composition, whether the antigen is native or recombinant, and the stability of the antigen. While initial testing will be done in a "clean" system where the antigen is spiked into a buffer or an artificial sample medium, switching to clinical samples as soon as possible is desirable and is discussed later in this handbook. Access to clinical samples is vital for effective assay development and validation.

It is important to remember that the development process is assay dependent. The strategy used for one assay may not be the appropriate strategy to use for another assay. The guidelines below are intended to provide a general overview of considerations when developing an LFA. Whether the assay is being developed as a qualitative, semi-quantitative, or quantitative assay is one of the most critical factors that will affect the significance of each of these development steps. At Fortis, we have experience with assays in both competitive and sandwich formats and in qualitative and quantitative platforms.

Lateral Flow Assay Design

An overview of a typical small-scale LFA manufacturing process at Fortis is shown in **Figure 6**. Small-scale production runs use backing cards that are 30 cm long and can be cut into the desired width, usually between 3-6 mm per strip.
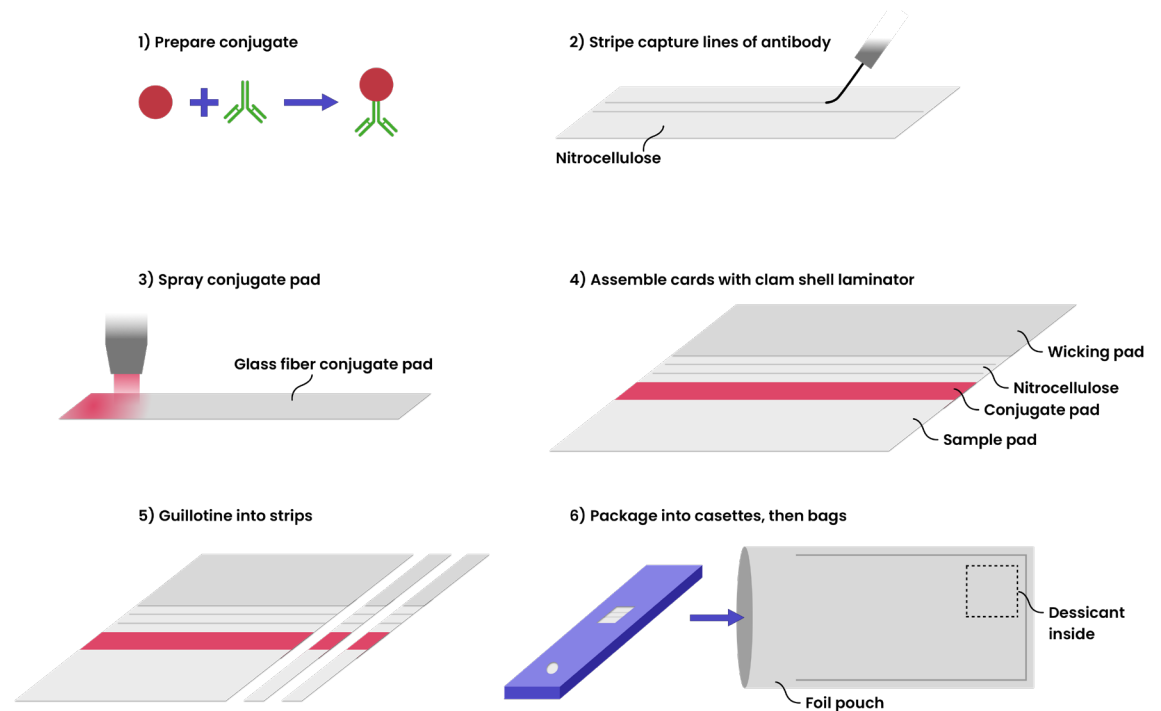

Figure 6: General procedure for the card-based assembly of a lateral flow device consisting of (1) conjugate preparation, (2) striping of capture lines, (3) spraying conjugate pad, (4) assembly of cards, (5) strip cutting, and (6) packaging into cassettes.

The first step in the process is to prepare conjugates (nanoparticle + antibody) and transfer the conjugates to an appropriate solution for spraying and drying onto the conjugate pad (**Figure 6, Step 1**). For reproducible striping of test and control lines, a dispenser with a flexible hollow glass or PEEK fiber connected to a syringe pump is used (**Figure 6, Step 2**). The conjugate is applied to the glass fiber conjugate pad using a non-contact spray head on the dispenser (**Figure 6, Step 3**). Once the solutions are applied to both the nitrocellulose membrane and the conjugate pad, these components are dried and cured in an oven at 37 °C. After drying, the nitrocellulose, conjugate pads, sample pad, and wick are transferred to a dry room (<20% RH) where they are assembled onto an adhesive backing card using a clamshell laminator. The laminator ensures the accurate placement of components onto the adhesive backing card with the correct overlaps and controlled application pressure. These fully laminated backing cards are referred to as "master cards". To assemble a master card, a nitrocellulose membrane is applied first, followed by the conjugate pad, wick pad, and then sample pad (**Figure 6, Step 4**). The assembled cards are then cut into individual strips with an automated guillotine (**Figure 6, Step 5**), and assembled into a plastic cassette that is sealed in a foil pouch with desiccant (**Figure 6, Step 6**).

The steps above describe a typical method for preparing a lateral flow test strip. In general, the design of a lateral flow assay is extremely flexible and can be tailored to a specific instrument or intended use. In any configuration, many optimization steps are required to successfully develop a lateral flow assay. The section below outlines these "steps" for development, but it is important to understand that lateral flow assay development is not necessarily a stepwise process. Most of these steps need to happen concurrently and will need to be revisited multiple times throughout the development cycle.

Step #1: Nanoparticle Selection

A critical step in lateral flow assay development is selecting the appropriate nanoparticle probe. Selection of the nanoparticle probe for lateral flow assay development should be based on the assay format, assay matrix, sensitivity requirements, and development team experience. At Fortis, we have developed the BioReady product line to simplify nanoparticle probe selection. The BioReady product line includes a variety of nanoparticles that have been specifically optimized to bind biomolecules to the surface and have been extensively tested in a wide range of lateral flow assays. The following section discusses the various BioReady products broken down by surface chemistry/conjugation strategy and particle size to further inform nanoparticle selection.

Nanoparticles for Passive Conjugation – Citrate Surface

BioReady gold nanoparticles with citrate surface are the appropriate choice when a passive conjugation strategy is desired. The "bare" particle surface is stabilized with weakly-associated citrate molecules that can be easily displaced in the presence of larger macromolecules like antibodies or proteins. Over time, the protein adsorbs to the surface of the nanoparticles through weak interactions such as van der Waals and/or ionic forces. Passive conjugation is the most commonly used conjugation technique and requires minimal chemistry.

Currently, the BioReady product line includes 40 nm and 80 nm gold nanospheres and 150 nm gold nanoshells with a citrate surface.

Advantages of passive conjugation:

- Traditional method of conjugate preparation

- Simple procedure with minimal chemistry involved

- Fewer parameters to optimize (can also be a disadvantage)

Disadvantages of passive conjugation:

- pH sweep required for adsorption optimization for each protein

- Proteins may desorb from the gold surface

- Risk of aggregation if conditions are not optimized

- High antibody loading required for optimal sensitivity

- Decreased stability in the presence of surfactants and/or extreme pH

40 nm Bare Gold Nanospheres (Citrate): 40 nm bare gold nanospheres are the most frequently used nanoparticles for lateral flow applications. 40 nm gold nanosphere conjugates result in the traditional ruby red color at the test and control lines in the presence of appropriate concentrations of the analyte of interest. We frequently recommend starting with 40 nm gold nanoparticles when developing a competitive assay.

80 nm Bare Gold Nanospheres (Citrate): 80 nm bare gold nanospheres typically provide moderate sensitivity enhancement over 40 nm nanospheres, particularly in sandwich assays, due to the optical properties of the larger particle size. 80 nm nanospheres can be handled similarly to 40 nm nanospheres and result in a similar red color at the test and control lines. 80 nm nanospheres are the best choice when a red color test and control line are desired but there is a need to improve sensitivity beyond what is achievable using 40 nm nanoparticles.

150 nm Bare Gold Nanoshells (Citrate): As shown in **Figure 7**, BioReady gold nanoshells can significantly enhance the sensitivity of lateral flow assays in comparison to 40 nm gold nanoparticles because each nanoshell particle is significantly darker than each 40 nm gold nanoparticle. As a result, fewer binding events are required for a signal to develop.

Figure 7: BioReady 150 nm citrate gold nanoshells provide sensitivity enhancement in a SARS-COV-2 assay.

Gold nanoshells have the same gold surface as traditional 40 nm spherical gold nanoparticles, so only minor modifications to existing 40 nm gold protocols are required. However, since there are fewer particles per OD, higher OD of nanoshells per strip may be necessary to maximize sensitivity in comparison to 40 nm nanoparticles.

Nanoparticles for Covalent Conjugation

BioReady gold nanoparticles with carboxyl surface (-COOH) are the best choice when a covalent conjugation strategy is desired. Carboxyl groups on the nanoparticle surface can be covalently linked to proteins or other biomolecules with a primary amine via carbodiimide crosslinker chemistry. This chemistry produces a stable amide or peptide bond between the nanoparticle surface and the biomolecule, resulting in a robust conjugate.

Currently, the BioReady product line includes 40 nm and 80 nm gold nanospheres and 150 nm gold nanoshells with a carboxyl surface. The BioReady product line also includes 40 nm gold nanospheres and 150 nm gold nanoshells with an NHS-activated surface, eliminating the need for the user to perform the intermediary EDC/Sulfo-NHS chemistry steps.

Advantages of covalent conjugation:	Disadvantages of covalent conjugation:
• Irreversible amide bond formed between the biomolecule and the gold surface	• Requires additional step to activate the particle surface via EDC/s-NHS chemistry
• Increased conjugate stability in a wider range of diluents and matrices (e.g. increased stability in surfactant-containing diluents and across a wider range of pH)	• EDC/sulfo-NHS chemicals and chemistry are sensitive and have specific handling requirements
• Greater control over protein/antibody loading	• Increased number of conjugation parameters to optimize
• Typically requires significantly less antibody/biomolecule loading	

40 nm Gold Nanospheres - Carboxyl: 40 nm gold nanospheres are the most frequently used nanoparticle size for lateral flow applications. The carboxyl group enables easy covalent conjugation to proteins (e.g., antibodies) and other amine-containing macromolecules. 40 nm gold nanosphere conjugates result in the traditional ruby red color at the test and control lines in the presence of appropriate concentrations of the analyte of interest. We frequently recommend starting with 40 nm gold nanoparticles when developing a competitive assay.

80 nm Gold Nanospheres – Carboxyl: 80 nm gold nanospheres – carboxyl typically provide moderate sensitivity enhancement over 40 nm nanospheres, particularly in sandwich assays, due to the optical properties of the larger particle size. 80 nm nanospheres can be handled similarly to 40 nm nanospheres and result in a similar red color at the test and control lines. 80 nm nanospheres are the best choice when a red color test and control line is desired, but there is a need to improve sensitivity beyond what is achievable using 40 nm nanoparticles.

150 nm Gold Nanoshells – Carboxyl: BioReady gold nanoshells can significantly enhance the sensitivity of lateral flow assays in comparison to 40 nm gold nanoparticles because each nanoshell particle is significantly darker than each 40 nm gold nanoparticle (**Figure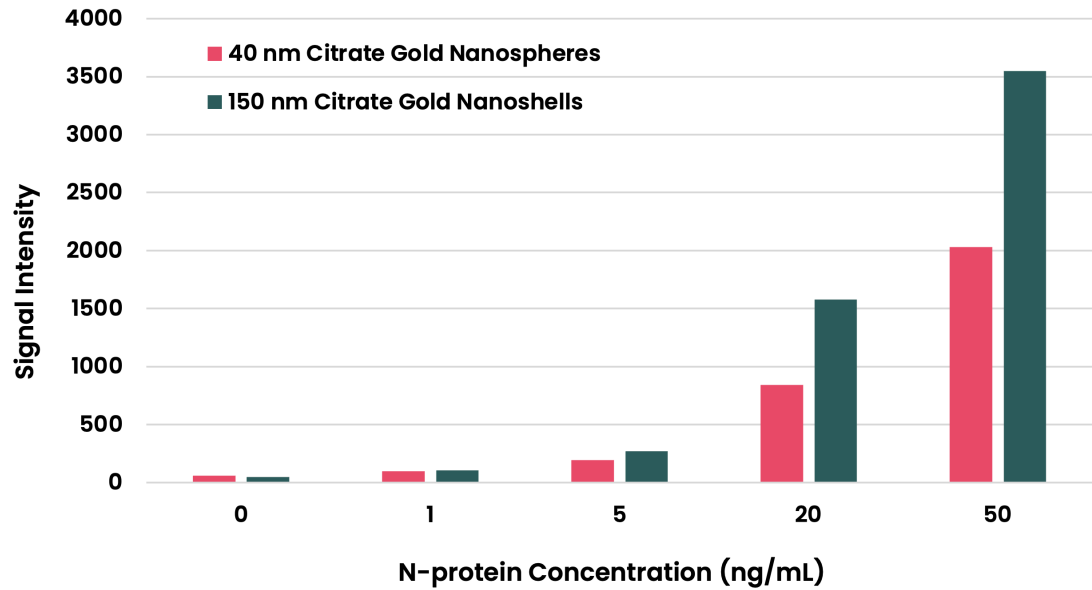 8**). As a result, fewer binding events are required to see signal development. However, since there are fewer particles per OD, a higher OD of nanoshells per strip may be necessary to maximize sensitivity in comparison to 40 nm nanoparticles.
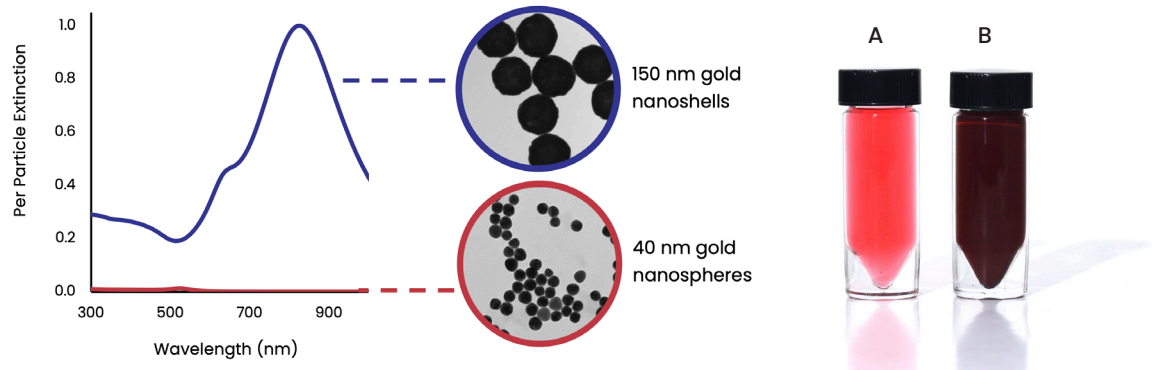
Figure 8: BioReady 150 nm gold nanoshells have a per particle extinction coefficient that is approximately 35-fold larger than 40 nm gold nanospheres, as shown in the graph (left) and photograph (right). The photograph shows a vial of 40 nm gold nanospheres (A) and 150 nm gold nanoshells (B) at the same particle number to demonstrate the difference in color intensity.

NHS-Activated Gold Nanoparticles

BioReady NHS-activated gold nanoparticles are an additional option when a covalent conjugation strategy is desired in a simplified procedure. The BioReady NHS-activated gold nanoparticles are surface functionalized with an active NHS ester, eliminating the need for the user to perform the intermediary EDC/Sulfo-NHS chemistry steps. The particles are supplied as a lyophilized powder that is simply resuspended with the provided reaction buffer and reacted with the desired protein/biomolecule to enable amide bond formation. Currently, the BioReady product line includes 40 nm gold nanospheres and 150 nm gold nanoshells with the NHS surface.

BioReady Gold Conjugates

The BioReady product line also includes several gold conjugates, including streptavidin, protein G, and anti-human IgG gold conjugates. These multi-purpose conjugates allow for relatively quick and easy binding to a variety of biomolecules without the need for extended optimization and are all covalently linked to gold nanoparticles.

Our best-selling conjugate is the streptavidin gold conjugate, this conjugate is currently offered as 40 nm gold conjugate and 150 nm gold nanoshell conjugate. Streptavidin gold conjugates can quickly and irreversibly bind to biotin-containing biomolecules, such as biotin-labeled antibodies or nucleic acids, for use in traditional and/or nucleic acid lateral flow assays.

Advantages of off-the-shelf gold conjugates:

- Stable, covalent conjugates for quick and easy binding to their respective targets

- Decreases conjugate development time

Disadvantage of off-the-shelf gold conjugates:

- More limited capacity for user optimization

Other Nanoparticle Probes

In addition to spherical gold nanoparticles, several other probes can be used in lateral flow assays. Dyed polystyrene particles and cellulose beads are commonly used but they can have high lot-to-lot variability, stability issues in select matrices, and challenges associated with optimization. For higher sensitivity, fluorescent probes like Europium beads are commonly used. However, fluorescent probes require specialized fluorescent readers to analyze the result, increasing the cost and limiting the use cases.

If you are unsure which probe is best suited for your application, please use the email below contact us. As a leader in nanotechnology, we excel at engineering custom nanoprobes to your specifications – please reach out to info@nanocomposix.com for additional information!

Step #2 Antibody Selection

Selection of the optimal antibodies is often the most critical step of lateral flow assay design. The performance of the lateral flow assays depends on the kinetics, affinity, and steric properties of the antibodies employed to bind to an analyte in the sample. Ultimately, it is these antibody binding characteristics that will determine the rapid visual readout seen by the end user. For a sandwich assay, two antibodies that can simultaneously bind to the target analyte with high sensitivity and specificity will be selected. In the case of a competitive assay, only a single antibody and target analyte conjugated to a carrier protein are needed.

Antibody Selection

Whether you are sourcing commercially available antibodies or relying on custom manufacturing, antibody selection is one of the most important steps in lateral flow assay design. Antibody cost, availability, sensitivity, specificity, kinetics, cross-reactivity, and antibody type (i.e., monoclonal or polyclonal) are all important factors when selecting an antibody. The first step in this process is to research what antibodies are available for your specific application target. The number of antibodies available for a specific analyte varies greatly, with some antibody vendors providing product information such as its application use in ELISA, Western Blot, or Lateral Flow. If antibodies are commercially available, aim at acquiring and testing as many as possible to perform the feasibility initial screening to determine which pairs are most effective. In the case of a competitive format assay, many analyte-protein conjugates should also be screened. If an antibody is not commercially available for the assay that you would like to develop, custom manufacturing of antibodies is an option. Custom antibody manufacturing allows for targeted immunization against the whole analyte, recombinant or native protein, or specific amino-acid sequences. Immunization of rabbit, mouse, or other host species typically yields multiple unique antibody clones irrespective of the immunization protocol. Custom manufacturing does not necessarily guarantee success in finding a clinically relevant antibody, but it does provide some valuable benefits later in development as well. Mainly, control of antibody supply to mitigate manufacturing risk, as well as a substantial decrease in cost when scaling up antibody production. Custom manufacturing does take time (weeks to months), which will have to be accounted for when deciding how best to achieve your assay development goals. When pursuing custom antibody development, it is important to consider the source, stability, and purity of the antigen used for immunization.

Typically, several monoclonal and polyclonal antibodies will be available for selection for the first round of screening. Monoclonal antibodies are preferred over polyclonal antibodies simply because they are at lower risk for manufacturing and have decreased lot-to-lot variability. In some cases, polyclonal antibodies may provide the best sensitivity due in part to their ability to bind multiple portions of the analyte. Monoclonal antibodies are assigned clone numbers, which indicates that the antibodies are from a single clone of hybridoma cells. Monoclonal antibodies with the same clone number may be available from multiple distributors so it is important to look at the clone number when selecting antibodies to avoid screening the same antibody twice. Other considerations for down-selecting antibodies are non-specific binding, cross-reactivity, the immunogen used for antibody development, specificity, sensitivity, and any pairing information that the supplier might have regarding the specific antibody. Cost is also a factor, as antibody costs can be a significant component of the bill of materials.

For antibody selection, it is best to screen the antibodies by building an initial version of a lateral flow assay that serves as a test platform. Antibodies perform differently in lateral flow than in formats such as an ELISA where the kinetics can be less important. In lateral flow, the antibody must remain active after being conjugated to the nanoparticles, retain its structural integrity when completely dried, and be instantly reactive upon rehydration by the sample. Traditional screening methods, such as ELISA or Western Blot may not meet all these requirements. Another notable difference is that these assays typically have long incubation times compared to lateral flow where the binding to the test line must occur in just a few seconds. Given the very short contact time, the kinetics of the antibody binding in lateral flow has a greater impact on the test result.

When choosing an appropriate antibody pair for a sandwich assay, we test every possible combination of the test line and conjugate antibody. For example, screening 4 monoclonal antibodies will result in 12 possible antibody configurations (**Table 1**). Due to steric hindrance and binding capabilities, an antibody pair that functions in one configuration may not work if the antibodies in the system are switched between the probe and the test line, so it is important to test both configurations. Additionally, conjugates prepared via passive adsorption may perform differently than when

conjugated via covalent methods. If switching from passive conjugation to covalent conjugation or vice-versa, it may be useful to re-screen antibodies conjugated in the intended format.

	Ab #1 on Particle	Ab #2 on Particle	Ab #3 on Particle	Ab #4 on Particle
Ab #1 at Test Line		×	×	×
Ab #2 at Test Line	×		×	×
Ab #1 at Test Line	×	×		×
Ab #2 at Test Line	×	×	×	

Table 1: Antibody pair evaluation matrix.

The advantage and disadvantages of polyclonal vs. monoclonal antibodies are listed in Table 2. Monoclonal antibodies are antibodies that have been propagated from a single cloned hybridoma, are structurally identical, and recognize a single epitope on an antigen. Monoclonal antibodies are also derived from single B-cell isolation post immunization, screening, and cloning. Polyclonal antibodies are a heterogeneous mixture of antibodies that potentially recognize multiple epitopes on an antigen. Affinity-purified polyclonal antibodies have been purified using an antigen specific column and this can lead to additional specificity in the assay as compared to crude polyclonal serum. Monoclonal antibodies are often preferred to conjugate to the nanoparticle because there is less variability between conjugations, they often have high specificity to the antigen, and they are less likely to cross link the nanoparticles. Polyclonal antibodies are preferred at the test line due to their high affinity and ability to recognize multiple epitopes. However, the lateral flow assay is not limited to this configuration. It is also possible to have two different monoclonal antibodies in the system that bind to two different epitopes on the antigen. Sometimes a polyclonal antibody is used both on the particle and the test line, although this is not ideal for quantitative assays. As with most other aspects of lateral flow development, empirical testing is the best way to determine the optimal conditions for each assay.

	Polyclonal	Monoclonal
Advantages	• Inexpensive to produce • High affinity • Recognize multiple epitopes (generally provides more robust detection) • Polyclonal antibodies are often preferred for the detection of denatured proteins • Higher tolerance for differences in antigen (i.e., glycosylation of proteins)	• Constant and renewable source • Consistency between lots • Less background relative to polyclonal antibodies • Homogeneity ensures reproducible results • Specificity of monoclonal antibodies make them extremely efficient for binding antigen within a mixture of related molecules
Disadvantages	• Prone to batch-to-batch variability • They produce large amounts of non-specific antibodies which can result in a background signal in some applications • Multiple epitopes make it important to check for cross-reactivity	• Monoclonal antibodies may be too specific (e.g., less likely to detect across a range of species)

Table 2: Advantages and disadvantages of using polyclonal and monoclonal antibodies.

Before finalizing the antibody selection, it is important to perform cross-reactivity experiments to ensure that the selected antibodies are not recognizing other analytes that will be present in the clinical sample. Because so much work goes into the subsequent optimization of the lateral flow test with the selected antibodies, potential cross-reactivity should be evaluated as early in development as possible.

Antibody Purification

For the conjugation of antibodies to nanoparticles, it is critical that the antibody is in the correct buffer. For passive adsorption of antibodies to nanoparticles, the buffer needs to be free of additional stabilizing proteins (e.g., BSA) and salt preservatives (e.g., sodium azide). The pH of the buffer should be optimized to improve the efficiency of conjugation. For covalent conjugations, the antibody buffer needs to be free from amines other than those on the protein (e.g., sodium azide, Tris buffer) and any additional stabilizing proteins. These molecules will compete with the amines in the antibody for conjugation sites. For best results, the antibody for conjugation should be purified and adjusted to a concentration of 1 mg/mL or greater in a low ionic strength buffer. We recommend 10 mM potassium phosphate. Antibodies can be purified and transferred into an amine-free buffer using spin columns or dialysis tubing with the appropriate molecular weight cut-off.

To purify antibodies from additional stabilizing proteins, an affinity column such as a protein A or G column is required. Since most protocols for separation with affinity columns use Tris as a buffer, subsequent purification is still necessary to remove free amines after the antibody is recovered. Whenever possible, obtain antibodies without any additional stabilizing proteins.

After protein purification, the concentration of the antibody should be verified to ensure that the correct amount of antibody is being conjugated to the nanoparticle. There are several ways to measure protein concentration including absorbance at 280 nm, a BCA assay, or a Bradford assay.

Control Line Antibody

In both sandwich and competitive assay formats, it is important to incorporate a second line on the membrane that functions as an internal quality or procedural control. The line will be visible in the presence or absence of an analyte and shows the end user that the assay is functional and that the results are valid. The control line antibody is typically a secondary antibody specific to the species of the conjugated antibody. For example, a mouse monoclonal antibody is often used as the antibody conjugated to the nanoparticle. In this system, a secondary antibody that is specific for a mouse antibody (i.e., goat anti-mouse) will bind the conjugated antibody in the presence or absence of an analyte and result in a visual readout. If the conjugate antibody is from a different species, the secondary antibody used at the control line needs to be specific for that species.

Step #3: Nitrocellulose Membrane

The nitrocellulose membrane is a critical component that contains the test and control line reagents and provides the readout of the assay results to the end user. During development, it is important to select the correct membrane type and optimize the striping parameters of your test and control line reagents to achieve the desired results.

Material & Type

Nitrocellulose membranes are available in various grades and porosities that wick an applied liquid sample at different speeds. Many manufacturers label their various grades based on the capillary flow time, which is the amount of time (seconds) required for the solvent front to advance 4 cm, With a fast nitrocellulose such as Millipore HF75, the solvent front progresses by 4 cm in 75 seconds. In a slow nitrocellulose, such as Millipore HF180, it takes 180 seconds (2.4 times longer) to cover the same distance. Some manufacturers may also label their grades in pore size (μm), which is directly related to the capillary flow time. A larger pore size correlates with a faster membrane (lower capillary flow time), and a smaller pore size correlates with a slower membrane (higher capillary flow time). Using a slower membrane (smaller pore size/higher capillary flow time) will increase the assay time. Slow speeds increase the incubation time between the nanoparticles, the analyte, and the test line, which in turn can increase the sensitivity. Faster membranes (larger pore size/lower capillary flow time), reduce the incubation time between the reagents in the system and yields a faster result (**Table 3**). Viscous samples (e.g., saliva, undiluted plasma, or solubilized solids) migrate slower than non-viscous samples such as urine and may flow better using faster membranes.

Membranes are available from several manufacturers including MDI, EMD Millipore, Cytiva, and Sartorius. Some membranes are treated with proprietary mixtures of surfactants and other chemicals to make them hydrophilic to aid the binding of protein to the membrane. Manufacturers such as MDI and Cytiva include product information on whether the material is low, medium, or high protein

binding. In addition to investigating the effects of capillary flow time/pore size on your assay, it is important to screen membranes for each assay from a variety of manufacturers. It is also important to note that the synthesis of these nitrocellulose membranes can be variable, so an examination of lot-to-lot variability should always be performed to ensure your assay performs similarly between lots.

Relative Flow Time	Relative Pore Size	Relative Sensitivity	Examples
Fast	Large	Low	• Millipore: HF75, 90 • Sartorius: CN95 • MDI: NC 15 µm • Cytiva: AE98, AE99
Medium	Medium	Medium	• Millipore: HF120, 135 • Sartorius: CN140, 150 • MDI: NC 8 µm • Cytiva: FF120 HP
Slow	Slow	Slow	• Millipore: HF180 • MDI: NC 5 µm • Cytica: FF170 HP

Table 3: Relationship between flow time, pore size, and sensitivity.

Membrane Striping

Another step in the lateral flow assay design process is to dispense the test and control antibody lines onto the nitrocellulose membranes. At Fortis, we use an Imagene Isoflow dispenser (**Figure 9**) although there are several manufacturers of reagent dispensers for lateral flow products (e.g., Kinematic, Biodot) which may use contact or non-c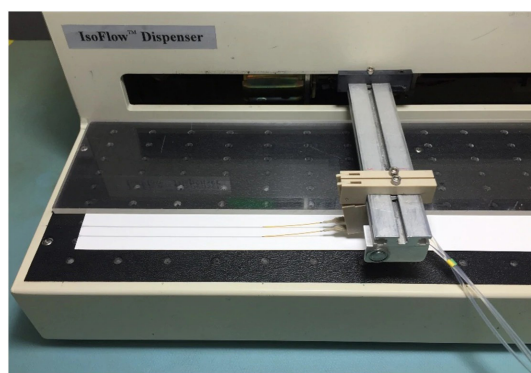ontact dispensing. The nitrocellulose membranes should not be stored in a desiccated environment prior to striping, but rather in a controlled humidity environment of ~50% relative humidity (RH). Nitrocellulose membranes that are too dry may result in spotty, non-uniform lines, while nitrocellulose that is too damp will result in a widened test line that may decrease the signal intensity. In principle, it is recommended to refer to the storage and handling guidelines provided by the manufacturer and adhere to their expiration dates. Overnight acclimation of the nitrocellulose membrane in a humidity-controlled environment is standard practice to ensure the membrane will be striped consistently. Once the nitrocellulose has been striped and dried, the humidity must be kept constant and low (less than 20% RH) until the test strips are sealed in pouches with desiccant. Exposure of the membranes to moisture after drying can interfere with protein stability and functionality.

Figure 9: Striping the nitrocellulose membrane with test and control solutions using an Imagene Isoflow dispenser.

Important parameters for striping the membrane include the reagent concentrations, dispense rate, dispense speed, and striping buffer which will all be dependent on the specific assay reagents and the membrane being used. Typical dispense rates using a contact dispenser are between 0.5 and 1 µL/cm which will result in a line width of approximately 1 mm. For medium and slow membranes, a dispense rate of 1 µL/cm is recommended. The larger pore size of the fast membranes will allow the solution to spread further and result in a wider line, so a decreased dispense rate (i.e. 0.8 µL/cm) is needed

to achieve the same line width. For competitive assays, an analyte-protein conjugate is dispensed at the test line rather than an antibody (e.g., drug of abuse-BSA or peptide-BSA). Analyte-protein conjugates tend to spread out more than antibody solutions, so the dispense rate may need to be decreased even further to obtain the same line width (0.5 µL/cm). The dispensing speed typically used for the Isoflow dispenser at Fortis is 20 mm/s. The width of the test line in LFAs can be critical to meet sensitivity requirements. When depositing the capture reagent on the membrane, concentrating the capture reagent with a tighter line width can in turn concentrate the color intensity for the same number of binding events. It may be of interest to optimize dispense rates and reagent concentrations.

For sandwich assays, 1 mg/mL is a recommended starting point for test and control line antibody concentrations but can range from 0.5 to 2 mg/mL. The concentration will depend on the sensitivity requirements and the affinity of the antibodies to the analyte in the sample. It is important to note that for some competitive assays, it may be necessary to stripe the test line at a concentration much lower than this (e.g., 0.1 mg/mL) to reach the desired dynamic range.

For any lateral flow assay, the buffer in which a particular protein is striped can have pronounced effects on the end results. To begin with, 1x PBS may work for most proteins, but some can be very sensitive to pH, salt concentration, and the presence of a stabilizer (e.g., trehalose). If challenges are encountered with stability, non-specific binding, signal strength, or kinetics, it may be worth evaluating the impact of the aforementioned buffer characteristics.

After striping the membranes, it is important to mark each membrane with the line location and orientation of the test and control lines. Although this may seem trivial, it will ensure that the membrane will be placed in the correct orientation when assembling test strips. It is also important to mark any sections of the membrane where striping may have been inconsistent (possibly from an air bubble in the line) so that these strips can be identified and discarded (refer to Step #7 below for strip assembly). After striping, it is recommended that membranes be cured in a forced-air convection oven. The curing, and subsequent overnight drying in a low humidity environment (i.e., <20% relative humidity environment), is important to fixing the antibodies to the nitrocellulose membranes. Exposure to moisture at any point after striping may cause the stability of the proteins on the membranes to be compromised. We recommend curing for 1 hour at 37 ºC in a forced-air convection oven, followed by overnight storage in a desiccated environment. Final packaging should also include desiccant pouches to ensure the nitrocellulose membrane is not exposed to any moisture. Optimizing the membrane handling post striping will be important to develop an efficient manufacturing process. The temperature and duration of the curing step, the length of time drying in a low humidity environment, are parameters to examine before finalizing a striping SOP.

An additional membrane-blocking step may be incorporated into the assay design and can aid in improved flow, stability of the test strip, reproducibility, and blocking non-specific binding to the nitrocellulose. Blocking buffers can include sugars, polymers, proteins, and/or surfactants and are applied after the membrane is striped and dried. While some developers may utilize this step, it can be time consuming during the optimization process and can add unnecessary steps when manufacturing at scale. Most nitrocellulose membranes are treated by the manufacturer with a proprietary solution in order to make the membranes hydrophilic. Blocking the membrane may wash these reagents off, so any membrane treatment must be carefully evaluated. Alternatively, the chemicals utilized to enhance performance may be incorporated into other parts of the test strip, such as the sample pad, conjugate pad, or running buffer which is outlined below.

Consistent membrane striping is critical for achieving reproducible lateral flow results. If you do not have access to a reagent dispenser, we offer membrane striping as a custom service. Contact us at info@nanocomposix.com for more details.

Step #4: Conjugate & Conjugate Pad

Optimizing the conjugation parameters is one of the key factors in creating a functional and effective assay. Once a conjugate is prepared, it will be dispensed and dried onto a conjugate pad. The conjugate pad contains the dried down nanoparticle-antibody conjugates for detection of your target analyte, so it is important to choose a conjugate pad material and treatment that maintains the integrity of the conjugate upon drying and long-term storage, and that releases the conjugate completely after wetting with the sample media.

Conjugation

Antibody-nanoparticle conjugates can be prepared via passive adsorption or covalent binding. When utilizing passive absorption, a pH titration is required, normally followed by a salt stability test to determine the antibody loading and pH conditions that maximize stability. For covalent conjugation, amide bond formation is always optimal between pH 7–7.4, so an initial conjugate is relatively easy to fabricate. General protocols for conjugation are available on our website, and optimization steps are listed in Step #10 "Assay Optimization" section in this document.

- BioReady 40 nm bare gold: passive adsorption

- BioReady 40 nm carboxyl gold: covalent conjugation

- BioReady 40 nm nhs gold: simplified covalent conjugation

- BioReady 150 nm carboxyl gold nanoshells: covalent conjugation

- BioReady 150 nm nhs gold nanoshells: simplified covalent conjugation

Performing a successful conjugation of antibodies to nanoparticles is critical in developing a functional assay and can require many rounds of optimization to enhance the efficacy of conjugation or improve the specific binding function of the conjugate. At Fortis, we have extensive experience in bio-conjugation to nanoparticles. We can help at any stage in the process from providing particles, protocols, and technical support, to optimization of custom conjugates that can be provided as a solution or dried down onto a conjugate pad. Refer to the assay optimization section below for more details on optimizing covalent conjugation conditions using BioReady particles.

Conjugate Stability & UV-Vis

Colloidal stability is incredibly important when designing and optimizing a lateral flow assay. A simple method for evaluating successful conjugation and conjugate stability is to measure and compare the UV-vis spectra before and after conjugation.

Gold nanoparticles absorb and scatter light with extraordinary efficiency and have unique optical signatures. Their strong interaction with light occurs because the conduction electrons on the metal surface undergo a collective oscillation when they are excited by light at specific wavelengths. This oscillation is known as a surface plasmon resonance (SPR) Gold nanoparticle absorption and scattering properties can be tuned by controlling the particle size, shape, and the local refractive index near the particle surface.

The effects of conjugation on optical properties: After a successful conjugation, there is a change in the local refractive index which can be observed in the UV-vis spectra as a distinct red-shift in the UV-vis spectra. In **Figure 10**, you can see normalized UV-vis spectra (each λ divided by λmax) of 80 nm gold, and 150 nm gold nanoshells (respectively) before and after conjugation. Notice that there is a uniform 2-3 nm red shift at the peak in the spectra around 550 nm for the 80 nm gold and 850 nm for the 150 nm gold nanoshells, but the overall shape of the spectra remains the same before and after conjugation. Additionally, another peak can be observed at 280 nm, which is arising from the excess protein in the conjugate diluent/storage buffer.
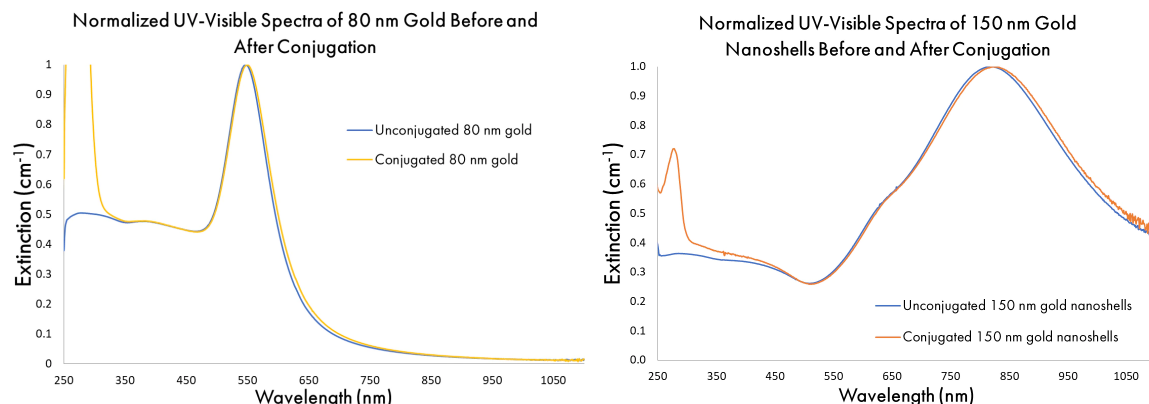
Figure 10: (Left) Normalized UV-vis spectra of 80 nm gold before and after successful conjugation, corrected by dividing the entire spectra λmax, shows any changes in shape of the UV-visible spectra. (Right) Normalized UV-vis spectra of 150 nm gold nanoshells before and after successful conjugation.

The effects of flocculation and aggregation on optical properties: When gold nanoparticle solutions are destabilized, they can flocculate (reversibly clump together) or aggregate (irreversibly clump together) rather than remain in a dispersed colloidal suspension. The optical properties of gold nanoparticles change when particles aggregate and the conduction electrons near each particle surface become delocalized and are shared amongst neighboring particles. When this occurs, the surface plasmon resonance shifts to lower energies, causing the absorption and scattering of all wavelengths to red-shift which creates an elevated baseline at longer wavelengths in the UV-Vis spectra (sometimes with a broadened peak).

UV-Visible spectroscopy can be used as a simple and reliable method for monitoring the stability of nanoparticle solutions. As the particles destabilize, the optical density (OD) will decrease due to the depletion of stable nanoparticles, and often the peak will broaden, or an elevated baseline or secondary peak will form at longer wavelengths (due to the formation of aggregates). In **Figure 11**, we plot the UV-vis spectra of a stable 80 nm gold conjugate, and an unstable 80 nm gold conjugate in two ways: A normalized UV-vis spectra (corrected by dividing the entire spectra to λmax) helps to observe any changes in the shape of the UV-visible spectra. A dilution-corrected UV-vis spectra allows us to look for changes in absorbance or optical density. **Figure 12** demonstrates the same observations but with 150 nm gold nanoshell conjugates.

Figure 11: (Left) Normalized UV-vis spectra, corrected by dividing the entire spectra λmax, shows any changes in shape of the UV-visible spectra. (Right) Dilution-corrected UV-vis spectra helps to look for changes in the absorbance or optical density. UV-vis spectra of a colloidally stable 80 nm gold conjugate and an 80 nm gold conjugate with poor colloidal stability.

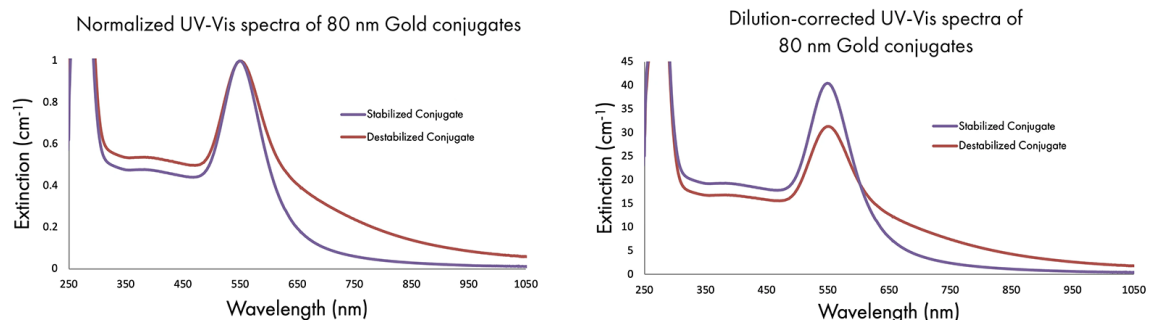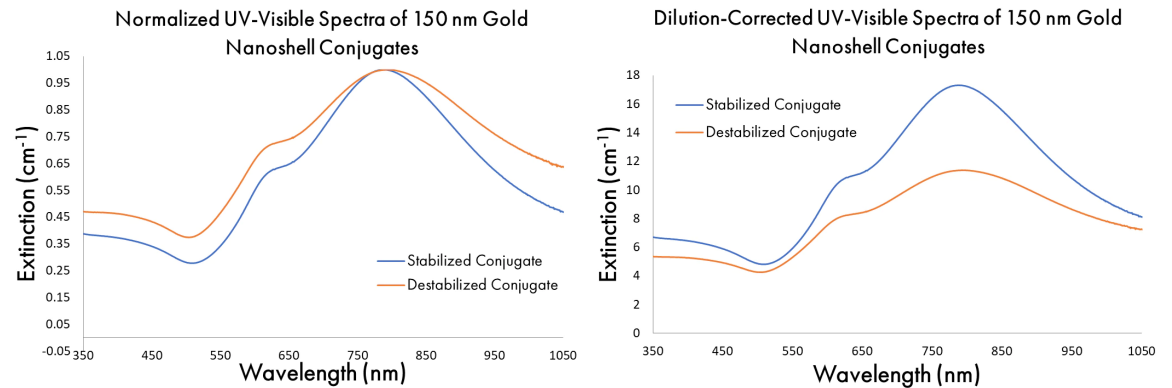**Figure 12:** UV-vis spectra of a colloidally stable 150 nm gold nanoshell conjugate and a 150 nm gold nanoshell conjugate with poor colloidal stability. (Left) normalized UV-vis spectra, corrected by dividing the entire spectra λmax, shows any changes in shape of the UV-visible spectra. (Right) Dilution-corrected UV-vis spectra helps to look for changes in the absorbance or optical density.

Conjugate Pad Material & Treatment

There are many conjugate pad materials available from multiple suppliers, such as Millipore, mdi, and Ahlstrom. Conjugate pads made of glass fiber are generally recommended but other materials may work well. The material will determine the volume of conjugate that can be absorbed, as well as the speed of release. Ahlstrom 8950 is a relatively low-density glass fiber and is a good starting material when the conjugate is to be dispensed at a low rate (<6 mL/cm) and needs to be released quickly. A fast release rate is often beneficial for competitive assays and for viscous sample mediums, such as saliva. Ahlstrom 8980 and Millipore GFDX are more dense glass fiber materials that can hold a relatively larger volume of conjugate and have a slower release rate. Conjugate capacity and release rate can improve sensitivity by increasing the number of conjugated particles and allowing a longer incubation time of the conjugated antibody and analyte in the sample. Along with most steps of the lateral flow development process, it is important to screen as many materials as possible for each assay to identify the most effective material for your specific application.

It may be necessary to pre-treat the conjugate pads before dispensing conjugate. The pre-treatment components often include a buffer for pH adjustment but should not contain a high concentration of salt as this may aggregate the nanoparticle conjugate. Proteins, polymers, and detergents can be added to the conjugate pad pre-treatment to aid in the release of the conjugate and flow of the assay. When running a test, these components move up the strip more quickly than the conjugate and can help block the membrane prior to the conjugate interaction. This reduces non-specific interactions. Adding blocking reagents and non-ionic surfactants to the dispensing conjugate solution can eliminate the need for blocking the membrane for simplified manufacturing.

Conjugate pad treatment can be performed by immersion, or by spraying uniformly with an automated dispenser. Materials should be dried in a forced air convection oven at 37 °C for 1-2 hours, and then cured and stored in a desiccated environment (<20% relative humidity) at 18-25 °C.

Drying Conjugate Onto Conjugate Pad

The conjugate is typically applied to the conjugate pad using an air jet dispenser. Several machines with hollow fiber dispensers used to stripe nitrocellulose membranes can also be configured with an air jet spray apparatus to dispense the conjugate onto the conjugate pads (e.g., Isoflow, BioDot, and Kinematic). Conjugate can also be applied to a conjugate pad by immersing the pad into the conjugate solution followed by drying. This method is only recommended if an air jet is not available, and the method has been optimized to provide valid results. Immersion into the conjugate solution does not allow for control over the conjugate volume, which is critical in many assays, especially for semi-quantitative or quantitative assays.

The buffer for the conjugate requires sugars to ensure the long-term stability of the dried conjugate and re-solubilization upon interaction with the sample. A recommended starting concentration of sugars for 40 nm gold and 150 nm gold nanoshell conjugates between 10-20 OD is 10% sucrose and 5% trehalose, although this should also be optimized to improve, flow, stability, and test results.

A typical starting dispense rate is 10 µL/cm of 40 nm Au at OD 10 or 15 µL/cm of 150 nm gold nanoshells at 20 OD. Although this is a starting point, the optimal dispense rate and OD can vary dramatically depending on the assay. After dispensing the conjugate, the conjugate pads are dried in a forced-air convection oven for 1 hour at 37 °C. The dried conjugate pads are cured overnight in a desiccated environment with <20% humidity prior to testing.

Step #5: Sample Pad Selection

The sample pad is the first material that comes in contact with the sample when running a lateral flow assay so the sample pad material and pre-treatment, if needed, should be evaluated to ensure that the sample has the optimal absorption, release, flow and, if necessary, pretreatment before it reaches the rest of the other components in the system. Although sometimes the same material used as a conjugate pad can function as a sample pad their function and geometries are different. Here are some of the main physical properties to evaluate in sample pads:

- Composition

- Thickness

- Liquid absorption

- Wicking rate

Sample pads can be made from various materials such as glass fiber, cellulose, cotton, and synthetic material. It's important to understand that the thickness of the sample pad may impact how the cassette housing the strip closes, and in turn, how sample flow in the cassette is affected. The added pressure from the cassette around the sample port may either completely block or slow down sample migration. The absorption capacity (measured in mg/cm^2) is an important product specification as it dictates the sample volume that can be loaded per test, usually, vendors will publish this information. The sample matrix of the assay should be considered when deciding which materials will be screened for development. It is always recommended to evaluate as many materials as possible to obtain the best results. Many of the materials, particularly the materials that will act as a filter for the sample, will have a "sidedness." Usually, the side that has a rougher texture will face up, while the smoother side will be face down. However, obtain as much information as possible from the supplier for the best use of the materials, and always screen and test the materials empirically to determine the best results.

In some sample matrices, such as urine and saliva, the composition of the sample can vary significantly depending on the individual, the time of day the sample is collected, food and drink consumed before sample collection, as well as many other biological factors. Chemical pretreatment of the sample pad before building the strip using optimized buffers can aid in "normalizing" the samples before they reach the conjugate. This may assist in sample solubilization or prevent any unfavorable interactions that may occur from the differences in pH, protein composition, mucins, and salt concentrations, that may cause non-specific interactions with the other reagents in the system (e.g., nanoparticles, affinity reagents) or particles in the system. For matrices such as whole blood and solubilized solids, it is critical to prevent the passage of unwanted material like red blood cells that may obscure the signal generated in the test line or control line. For these samples the sample pad can act as a physical filter preventing particulate material from reaching the nitrocellulose membrane while allowing the fluid containing the analyte of interest to flow through the test strip. In the case of whole blood, vendors have developed different sample pads with the ability to hold back red blood cells while allowing the plasma/serum and running buffer to pass through the rest of the strip at a rate compatible with the reading time of the test (**Figure 13**). It is also critical to understand whether the sample pad material has an affinity for the analyte as this will cause interference with the assay, effectively reducing sensitivity or leading to false negative results.

Figure 13: Lateral flow assay with sample pad that has a red blood cell filter. Image from mdimembrane.com

Treating the sample pad with an optimized buffer can enhance assay performance by mitigating sample variability (pH, viscosity, protein concentration, salt concentration, etc.), and improving flow and consistency of the assay. Treatment buffers can normalize the sample pH and salt concentration, act as blocking agents, improve flow, and enhance the reproducibility of the assay by incorporating proteins, surfactants, salts, and/or polymers at the appropriate concentrations. To determine what to include in the sample pad treatment, evaluate what aspect of the sample needs to be "normalized." For saliva samples, one challenge may be the difference in the viscosity of the samples. By incorporating salts and surfactants, the mucins and proteins can be broken down in turn decreasing viscosity and improving flow. However, if the sample is whole blood, the same components may cause hemolysis of the red blood cells and cause unwanted passage of cell fragments or hemoglobin through the membrane, this may obscure the test line or control line signal thus compromising the assay interpretation. In this case, gentler formulations are recommended, including lower concentrations of salts and detergents.

Sample pad treatment can be performed by immersion, by deposition with a pipette, or by spraying uniformly with an automated dispenser (e.g., Isoflow, Kinematic, Biodot). Spraying results in a more controlled result. After treating the sample pad, it should be dried in a forced air convection oven at 37 ºC for 1-2 hours and then cured overnight and stored in a desiccated environment (<20% relative humidity) at 18-25 ºC. Special care must be taken when handling pre-treated sample pads as the pretreatment may reduce their tensile strength and make them more fragile, this observation deserves special consideration when the test reaches the stage of large-scale manufacturing in which continuous breakage of the sample pad can occur.

Step #6: Absorbent Pad (Wick) Selection

The purpose of the wick pad is to absorb all the reagents that were not taken up by the test and control lines while maintaining capillary flow through the membrane to clear the background. The material and size of the wicking pad should be selected such that the absorption capacity is much higher than the sample and running buffer volume. Increasing sample volume can increase sensitivity; increasing running buffer volume can decrease non-specific binding due to washing of the test line, however, in both cases, a wick with a larger bed volume or absorption capacity must be selected to accommodate the increased volumes. Most wick pads are typically made of cellulose fibers due to their large bed volumes. A thicker material does not necessarily perform better or have a higher absorption capacity than a thinner material.

Wicking rate will also influence read time. The wick pad should prevent the backflow of the excess reagents for as long as possible, at a minimum, past the read time of the test. Eventually, the sample will diffuse back into the membrane, so it is important to characterize the stability window for the test line signal via the assay's kinetic profile. The correct wick pad selection will minimize the backflow of any excess reagents and provide a wide window for the time of assay readout.

Absorbent pad materials can be purchased from companies such as Millipore, Whatman/Cytiva, or Ahlstrom. Typical attributes that are reported for wick pads are the material, thickness, weight, wicking rate, and water absorption. In the end, different materials should be screened for the best functional results.

Step #7: Test Strip Assembly

The strategy chosen to assemble strips depends mainly on the required throughput. At larger scale manufacturing (e.g., >1 million tests per year), reel-to-reel dispense systems and automated laminators can be employed to facilitate production. At a smaller manufacturing scale and for R&D activities XYZ dispense systems, manual card lamination, or single card lamination systems are more simple and economical routes.
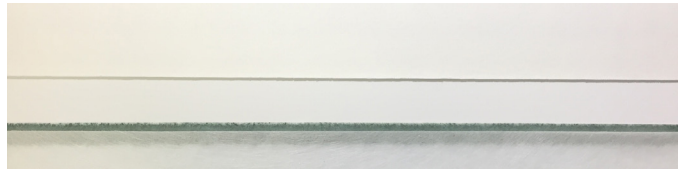

Figure 14: Assembled 30 cm master card with 150 nm gold nanoshell conjugate (see a dark green section at the bottom half of the card). This card is subsequently cut into the final test strips orthogonal to its length.

Fortis uses 30 cm backing cards from companies such as Lohmann, PDC, and Kenosha Tapes for assembling the lateral flow tests (**Figure 14**). These cards have an LFA-compatible adhesive coating underneath a series of peel-off layers, which are removed to attach the various components of the test strips (**Figure 6**). The nitrocellulose membrane, conjugate pad, sample pad, and wick pad can all be applied by hand or with a laminator.

The laminator registration key has customizable notches that specify exactly where each component will be placed and ensures reproducible assembly onto the adhesive backing card. Laminators greatly reduce the variability of component placement, in turn generating more reproducible test results with a lower variation.

Before test strips can be produced, master cards must be first assembled. The industry standard is to produce master cards 30 cm in length. Next, depending on each individual design, master cards are assembled with a sample pad, conjugate pad, nitrocellulose membrane, and wicking pad, finally, the master card is cut into strips ranging from 2.5 mm to 6 mm in width. Consistent strips are obtained when cutting is performed with automated guillotines (e.g., Kinematic, BioDot) that can be programmed for cutting strips of specific widths and in specific amounts. Thinner strips are more cost-effective (a higher number of strips per card can be obtained) but can be less accurate due to edge effects while strips for quantitative assays are most commonly cut to 5-6 mm widths. Special attention must be given to material overlaps, these overlaps are achieved by consecutively laying over all the materials: sample pad/conjugate pad, conjugate pad/nitrocellulose membrane, and nitrocellulose membrane/wicking pad. Overlaps in between construction materials along the strip can vary, from 2 mm to 5 mm. Importantly, overlaps can impact sample and conjugate flow, as well as the assay running time. In turn, this can affect assay sensitivity and specificity.

The cassette that houses the test strip can be one of the most critical components to achieving a reproducible and reliable assay, which is especially important for quantitative tests. The cassette provides optimal flow control by applying pressure at appropriate points along the strip ensuring that all the fluid passes through the strip assembly at the same flow rate. It also needs to ensure that the fluid flows through the test strip materials rather than flooding the strip or flowing along the edges. Typically, cassettes are designed after all materials have been selected and optimized and are customized to the lengths, widths, and thicknesses of each component. Control over strip pressure can control the flow rate of the sample fluid, allowing for longer or shorter incubation times of conjugate with the sample analyte. For large-scale production, a custom-designed cassette from an experienced industrial design company is necessary, preferably a company that already has lateral flow cassette expertise. When using a custom design, it is always important to keep in mind the mitigation of manufacturing risk downstream. This is an often overlooked, but critical component of the designing process. For initial testing, existing generic cassettes or 3D printing prototypes may be sufficient. Cassette design, design validation, and later manufacturing are expensive and lengthy processes, special attention must be given to timelines and budgets to reach the successful completion of these phases of assay development.

For some assays, cassetted strips are not required and a dipstick format may be the needed format to produce a fully functional assay. In this format, the strip is not placed inside a plastic cassette, instead cover tape may be used to provide flow control, protect the strip from physical damage, and hold all the components of the strip together. Cover tape is applied to the entire master card length and can extend from the wicking pad down to approximately 5 mm before reaching the end of the sample pad. The dipstick format is more economical since there is no need for a cassette, however depending on the application not every assay is amenable to this format. Commonly dipsticks are

used in the clinic with assays that use matrices such as urine or saliva, and in food analysis, to detect the presence of residual antibiotics in raw milk.

Step #8: Running the Assay

When you are ready to run your test strip, multiple methods can be used depending on where you are in the stages of development.

Dipstick Assay (Liquid Conjugate)

For the initial screening of antibodies, you can start by testing the conjugate in liquid format, in which the strip is prepared with a single pad instead of both a sample and a conjugate pad. The conjugate can be applied to the pad in liquid format, immediately followed by positive and negative samples to run up the strip. The strips can also be assembled as "dipstick" assays, in which there is no sample or conjugate pad. Instead, the sample and liquid conjugate are mixed in a well of a 96-well plate, or a small test tube (**Figure 15**). The strip is then dipped in the mixture and the solution is allowed to wick up the strip. For certain optimization steps, such as determining the appropriate dispense rate or pre-treatment buffers, dipstick assays can also be used. After screening initial parameters, it is important to test the strip in the fully assembled format with the sample pad and the dried down conjugate.
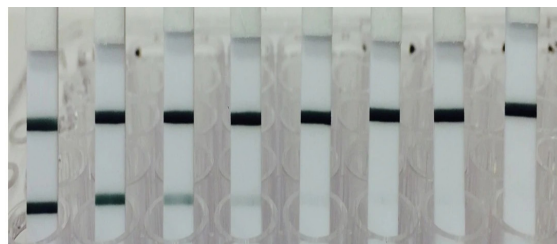

Figure 15: Running liquid conjugate in half strip format.

Full Assays (Dried Conjugate)

Once the conjugate is dried down and the strips are fully assembled (through Step #7), the test strips can be run by applying the appropriate amount of sample and/or running buffer to the sample pad and allowing the test to run. It is important to apply the correct volume to the test strip to ensure that there is enough liquid to initiate flow through the test while not applying an excess of liquid that "floods" the assay. Flooding occurs at the interface between the conjugate pad and the membrane when too much sample is applied to the sample pad. This will cause the fluid to run over the membrane rather than through the membrane and can negatively affect the test results. The volume of the sample to be applied will depend on several factors including the width of the test strip, the sample matrix, the material used as the sample and conjugate pad, and the overall flow characteristics of the strips.

Running Buffer

The running buffer can also be a critical factor in the lateral flow assay. The running buffer is a means of introducing critical reagents to the assay to help with buffering the sample pH, minimizing non-specific binding, neutralizing interferents, and increasing/decreasing flow speed. Most of these reagents can be introduced by other methods (see Steps #4 and #5), so a running buffer is not always required. One example where a running buffer is traditionally used is whole blood. In this case, a small amount of sample is added to the test strip, and then "chased" with a running buffer to run the assay. For each lateral flow assay, the buffer will need to be optimized for the individual assay by evaluating the buffering component, molarity, salt, detergent, polymers, and/or proteins. Always keep in mind that the simpler the running buffer is, the easier it will be to manufacture, and the longer the shelf life will be. 1x PBS with 1% Tween 20 is a good starting place for a running buffer, however, the formulation should be optimized for each assay.

Step #9: Analyzing the Strip

When analyzing the test strip, choosing the appropriate analysis method will depend on the stage of development and whether the assay is intended to be qualitative or quantitative. For effective optimization, it is important to assess the strips both quantitively as well as qualitatively. Unreleased conjugate from the conjugate pad, conjugate that has aggregated or bound to the membrane will negatively impact the conjugate available to be bound to the test line. These issues can be diagnosed by a well-trained eye. Next, an objective means of quantifying the output of the test strips is critical. The flow of the conjugate through the strip, the presence of any non-specific binding at the test line, and the intensity of binding at the test line when running a true positive sample are all important factors.

The first option is to read the assay by eye. This is acceptable for positive/negative scoring but is not useful for semi-quantitative or quantitative assays. A gradient scorecard can be used where the strength of the lateral flow line can be measured against a printed line intensity to give a semi-quantitative score by eye, this is useful for early assay development.

A flatbed scanner or a camera set up with controlled lighting can be used to capture an image of the test line. The color density (and thus line strength) can be analyzed in an image analysis program (e.g., ImageJ) resulting in a number that is directly correlated to the test line intensity. Various commercial readers are also available that will analyze strips. At Fortis, we use a Lumos camera-based reader or a ChemBio reader that provides a quantitative readout in approximately 30 seconds. Portable readers like the ChemBio Cube have become more common across the industry as have cellphone based photographic readers with accompanying apps. We also have several cellphone-based reader technologies in development.

Step #10: Assay Optimization

Establishing input requirements or assay performance specifications will be very important in providing a clear goal for the optimization process. Many components need to be meticulously optimized to develop a lateral flow assay that will successfully meet a specific need. The optimization process includes choosing the appropriate antibody pair, conjugation conditions, sample pad material and treatment, conjugate pad material and treatment, nitrocellulose membrane, test line concentration, wick pad material, running buffer, cassette, and sample volume, among other things (**Figure 16**). For quantitative assays, value-assignment, and traceability of calibrators as well as the elimination of matrix effects will also have to be carefully optimized.

1. Nanoparticle
- 40 nm gold for passive adsorption
- 40 nm gold for covalent binding
- 150 nm gold nanoshells for increased sensitivity

9. Analyzing
- Reader method
- Binding/sensitivity
- Flow

8. Running
- Sample volume
- Sample type
- Running buffer

7. Test Strip
- Card assembly
- Test strips
- Cassettes

6. Wick Pad
- Material
- Size
- Absorption capacity

5. Sample Pad
- Material
- Treatment
- Size

Assay Optimization

2. Antibody
- Test line antibody
- Detector antibody
- Control line antibody

3. Membrane
- Manufacturer
- Flow time/pore size
- Striping conditions

4. Conjugate
- Conjugation & dispensing
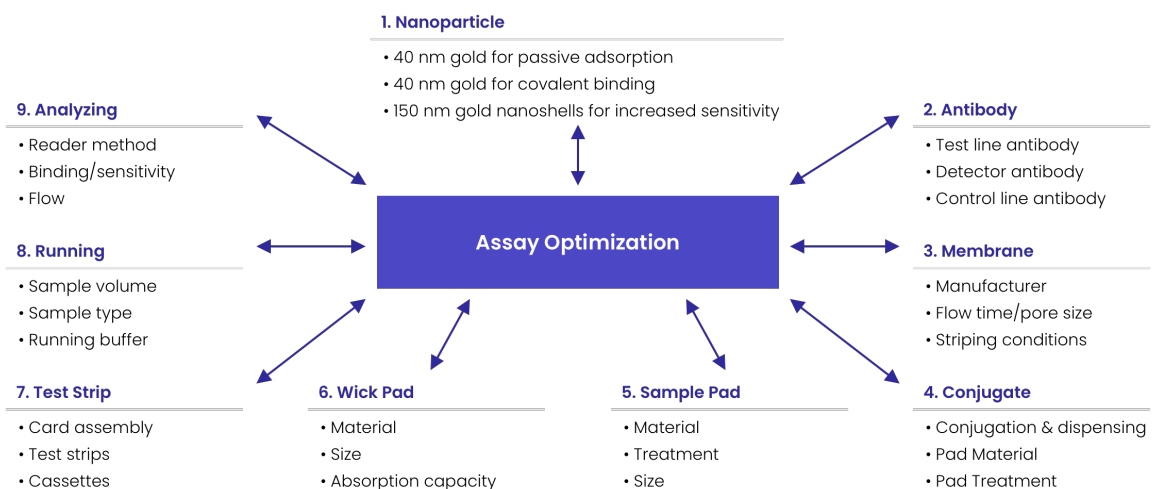- Pad Material
- Pad Treatment

Figure 16: Iterative optimizations that are performed to maximize performance of lateral flow assay. The steps outlined in this handbook are often revisited multiple times throughout the development cycle until the assay results meet your desired requirements.

There is an interdependence among all these components that need to be carefully balanced and oftentimes adjusting one variable will have unintended effects on another aspect of the assay. Accordingly, the development process is not linear, but iterative and very much empirical. For example, the formulation of the running buffer may be changed to introduce a harsher detergent to eliminate non-specific binding however it may also reduce the positive signal too much whereby sensitivity is negatively impacted. As a result, the concentration of test line antibodies may need to be increased to try to compensate for that. After each optimization step, prototypes of the latest best mode are manufactured, and testing is performed to understand the performance characteristics of the assay. The next optimization steps focus on improving those specifications by further tweaking the same variables or trying to change new ones. The process is repeated until the assay is refined enough to meet all design inputs.

As a rule, it is important to be as consistent as possible during the development cycle to reduce variation between conjugations and testing. Small details during the experimental protocols (e.g., spin speeds, spin times, pH, tube material, etc.) may seem trivial but could significantly affect the assay results downstream and confound results. A good experimental setup with changing only one or a few variables at a time, along with proper controls, can help with making sound decisions on the development pathway. Initial experiments can be performed in a "clean" system that is more artificial, to isolate problem components. For example, it might be acceptable to work with serum samples on bare strips when first developing a whole blood assay, to choose affinity reagents with the correct specificity. As development proceeds, however, optimizations should transition to using the final format of the product. In the case of the whole blood assay, that would mean testing in the final cassette with whole blood samples including a blood separator pad and the device that would transfer the sample onto the sample port. It is a good idea to periodically spot-check performance out in the field using alpha- or beta-tests. This can provide valuable feedback on where to focus further optimizations and sometimes reveal unexpected gaps. It can also be a reality check for the clinical application. Sometimes inputs are re-adjusted to compromise for a first-generation product (i.e., minimum viable product) that can be achieved more readily.

It should be noted that each assay, and even each antibody pair for the same assay, behaves differently. While some fundamental principles of cause and effect during assay development are followed, in the end, trying something out will often produce unexpected results (good or bad). Some assay developers employ a Design of Experiments approach (DOE) to aid in experimental setup and decision-making when multiple variables are being evaluated. Two critical output metrics to evaluate are increased signal intensity and elimination of non-specific signals, but performance requirements extend beyond just those two parameters (e.g., intra-assay CVs, inter-assay CVs, dynamic range, the limit of detection, shelf-life stability, etc.) Experience knowing which assay variables can have the biggest impact on performance plays a big role in efficient assay development and the likelihood of a successful product out the back end. This is where a contract development organization, like Fortis, which has decades of assay experience can help move a project along.

Conjugation: Tubes & Reagents

If you observe flocculation of your particles to the side of the tube (i.e. particles "sticking" to the walls) after centrifugation to remove excess EDC and Sulfo-NHS, the tube itself may be causing issues. Tubes that contain residual plasticizers or specialized tubes (e.g. low-bind tubes) may interfere with the particle stability and may cause this flocculation. We recommend using tubes manufactured by Labcon which are available through VWR and other distributors. If low-bind tubes are desirable, we recommend performing the activation step in Labcon tubes, and after the activated particles have been centrifuged and resuspended in the reaction buffer, the particles may be transferred to a low-bind tube prior to the addition of the antibody. Contact us for more information regarding the best tubes and procedures if this issue continues.

Conjugation: Conjugation Reagents

It is extremely critical to properly store and handle the EDC and Sulfo-NHS reagents to be used for conjugation. These reagents, particularly EDC, are sensitive to moisture and should be stored sealed, and desiccated according to the supplier's instructions. Because the reagents are sensitive to moisture, it is critical to bring them to room temperature before opening the vials and exposing them to the outside air. Remove the EDC and Sulfo-NHS from cold storage and bring them to room temperature for 45 minutes. Once at room temperature, open the vials and aliquot each reagent into a separate sealable container. Prepare fresh solutions immediately before conjugation. After

the reagents are aliquoted, seal the vials with parafilm and return them to the appropriate storage location. Follow our conjugation protocols for more information on the addition of EDC and Sulfo-NHS to activate the carboxylic acids on the particles. Single-use aliquots of EDC and Sulfo-NHS are available on our website and from many other vendors.

The pH of buffers is important for conjugation and performance of the conjugate. Always check the pH of the buffers immediately before use to ensure they are in the desired range.

Conjugation: Reaction Buffer

The reaction buffer should be evaluated for each antibody. For covalent conjugation to BioReady gold, our protocols use a reaction buffer of 5 mM potassium phosphate, 5 mg/mL PEG20 at pH 7.4. While activation of NHS esters is most efficient at ph 5, the reaction of Sulfo-NHS-activated molecules (NHS ester is the semi-stable intermediate formed during EDC/NHS coupling) is most efficient at pH 7-8. **NHS esters have a half-life of 4-5 hours at pH 7, 1 hour at pH 8, and only 10 minutes at pH 8.6.**

For optimal performance, we recommend investigating the following reaction buffers;

- 5 mM potassium phosphate 5 mg/mL PEG20 at pH 7.4

- 5 mM sodium phosphate 5 mg/mL PEG20 at pH 7.4

- 0.01x PBS 5 mg/mL PEG20 at pH 7.4

PEG 20 is included in the buffer to help redisperse the pelleted particles. It is not required but is recommended. Detergents such as 0.1% Tween 20 can be added to reaction buffers and may help stabilize conjugates during centrifugation steps.

Conjugation: Antibody Incubation Time

If you are not achieving the desired sensitivity or have encountered non-specific binding issues, the antibody incubation time and antibody-to-gold ratio can be evaluated. For covalent conjugations, our protocol recommends starting with a 1 hour incubation time. During optimization, shorter and longer incubations should be evaluated. In circumstances where you are limiting the number of antibodies per particle rather than saturating the surface (e.g. competitive assays) we generally recommend a shorter incubation time (as short as 5 minutes) before quenching to reduce the chances of antibodies folding and binding to several available acid groups on the surface and decreasing antibody functionality.

Conjugation: Antibody-to-Gold Ratio

Optimizing the antibody-to-gold ratio can significantly improve assay results by eliminating non-specific binding and increasing assay sensitivity. Covalent conjugation is a great method for controlling the amount of antibody per particle. We have effectively conjugated the carboxyl gold nanoshells with an antibody ratio of 2-30 mg antibody per 1 mL OD 20 particles and our 40 nm carboxyl gold with an antibody ratio of 2-60 mg antibody per 1 mL OD 20 particles. The optimal antibody ratio is determined by conjugating at various ratios and testing negative and positive samples empirically on the lateral flow strip. By evaluating stability, non-specific binding, and sensitivity levels, you can narrow your range and find the optimal ratio of antibody to particles. When decreasing the number of antibodies on the surface, it may be desirable to use a shorter incubation time, as mentioned above. Always be sure to quench (stop the reaction by adding a solution containing primary amines such as Tris, glycine, hydroxylamine, BSA, etc.) any remaining NHS-esters prior to processing to avoid crosslinking of particles.

Conjugation: Diluent Components

Conjugate diluent components will vary significantly between assays. Our covalent conjugation protocol recommends a diluent of 0.5X PBS, 0.5% BSA, 1% Tween 20, and 0.05% Sodium Azide at pH 8. While this has been shown to work well between different assays using our particles, we always evaluate the components individually and at different concentrations to determine the optimal conditions. The addition of casein (e.g. 0.5%), or titration of the other components (BSA 0-2%, Tween 20 0-2%, Triton X-100, Surfactant 10G, pH 7-9) can improve stability and assay results. Covalent conjugates

are more robust than passive adsorption conjugates and can remain more stable in the presence of detergents and other components. The conjugate diluent will most likely need to be re-evaluated when switching from a clean system (analyte spiked into the buffer) to the real sample media (i.e. saliva, urine, blood). The optimal formulation is tested empirically by running the conjugates in various diluents on the lateral flow assay and observing stability, non-specific binding, and sensitivity levels.

Frequently Asked Questions

Why is it important to purify my antibody from free amines such as sodium azide or tris buffers when performing a covalent conjugation?

Covalent conjugation with our carboxyl and NHS nanoparticles uses Sulfo-NHS esters that couple rapidly with amines on target proteins. Having other free amines in the reaction will compete with your target molecule for binding sites on the nanoparticle. We recommend purification using Amicon Ultra centrifugal filters as a quick and easy way to purify and perform buffer exchange (see more about antibody purification).

What are the advantages of carboxyl nanoparticles over nhs nanoparticles?

The NHS nanoparticles are a great tool for quickly evaluating antibody pairs – especially for small-scale "proof of concept" studies, or in lateral flow where it is critical to pair antibodies on a strip to mimic appropriate kinetic conditions. However, the NHS nanoparticles are limited by scale. The NHS-ester moiety hydrolyses in water. We rely on a quick lyophilization of the particles to 'pin' the NHS ester reactivity. Performing this process with large volumes of material slows down the process and reduces the amount of active NHS-ester on the surface of the particles.

What are the advantages of carboxyl nanoparticles over citrate nanoparticles (covalent conjugation over passive adsorption)?

Covalent conjugates are more stable than conjugates prepared by passive adsorption because the amide bond is permanent, and the antibody won't dissociate over time. Additionally, the covalent coupling procedure is not dependent on the isoelectric point of the antibody, removing the need for extensive pH sweeps saving time and reducing costs. Furthermore, the amount of antibody required per unit particle for covalent conjugates is often less than that required for passive adsorption.

Why is the buffer selection important when performing covalent conjugations?

While the conjugation pH is not dependent on the isoelectric point of the specific antibody, the pH for covalent coupling is still greatly important. The activation with EDC and Sulfo-NHS is most efficient at pH 4.5-7.2. The reaction of Sulfo-NHS-activated molecules (NHS ester is the semi-stable intermediate formed during EDC/NHS coupling) is most efficient at pH 7-8. NHS esters have a half-life of 4-5 hours at pH 7, 1 hour at pH 8, and only 10 minutes at pH 8.6

How can gold nanoshells increase sensitivity in lateral flow?

Our 150 nm gold nanoshells are 30 times visibly brighter per particle than traditional 40 nm gold used in lateral flow. Because they have been engineered with a silica core, they are twice as light as a solid 150 nm gold particle and flow easily through a nitrocellulose membrane. It is important to note that for any OD per volume, there are about 30× fewer nanoshells by particle number, so conjugate volumes will need to be adjusted appropriately) to maximize binding events. As a starting point, increasing OD or conjugate volume per strip two-fold will give you a boost in sensitivity.

What if I see false positive results?

When a test line is visible in the absence of the desired analyte, the false positive result may be caused by several factors such as non-specific binding, cross-reactivity, or heterophilic antibodies. To optimize the assay and eliminate the false positive result, it is important to understand which of these factors or a combination of factors is giving rise to a false positive result. Non-specific binding occurs when there is a non-specific interaction between the antibody-nanoparticle conjugate and the antibody at

the test line, regardless of the presence or absence of the target analyte in the sample. If this occurs, blocking agents such as proteins, surfactants, or polymers need to be incorporated in a component of the test strip (e.g. sample pad pre-treatment, conjugate pad pre-treatment, running buffer, conjugate diluent, etc.). Cross-reactivity is different from non-specific binding and occurs when the antibody has an affinity for an analyte in the sample that is NOT the target analyte. This issue is more difficult to address and usually will result in the need to change antibody systems that do not have cross-reactivity to unwanted analytes. The presence of heterophilic antibodies in a sample will result in a strong false positive result. There are multiple types of heterophilic antibodies that can cause a type of cross-linking between the antibody conjugated to the nanoparticle and the antibody at the test line, even in the absence of the target analyte. To test if your sample contains heterophilic antibodies, perform a serial dilution of the sample. If the false positive result remains strong even after diluting the sample instead of showing a linear decrease in signal intensity, it may be due to heterophilic antibodies. To prevent heterophilic interference, heterophilic blocking reagents are commercially available), or a mouse IgG conjugate can be added to the assay if the heterophilic antibody is specifically a human anti-mouse monoclonal antibody (HAMA).

Why do I see a decrease in the test and control line when switching from a "clean" (analyte spiked in buffer) system to a real sample (saliva, whole blood, etc.)?

A decrease or loss of a test and control line when switching from a "clean" system to a clinical sample may be due to the many additional components that exist in the clinical sample such as proteins, salt, or additional metabolites or molecules. The addition of blocking agents such as proteins, surfactants, or polymers into the conjugate diluent, conjugate pad pre-treatment buffer, or a running buffer can help recover the signal intensity.

What other factors can influence conjugation results?

If running under the correct pH conditions and the antibody incubation time has been optimized, confirm that EDC and Sulfo-NHS have been stored properly and that it is prepared just prior to conjugation. EDC should always be stored at -20ºC and Sulfo-NHS between 4-8ºC. It is important to allow reagents to come to room temperature prior to opening the bottles to avoid condensation from the atmosphere as both EDC in particular and Sulfo-NHS are moisture sensitive. For preparation, we recommend bringing bottles to room temperature for ~45 minutes before opening vials, weighing out a precise mass into a microcentrifuge tube, and then dissolving into a volume of water immediately before adding to the colloidal suspension.

Are your particles tolerant to detergents such as DMSO or Tween?

After stable conjugates are made, they are very stable with almost all detergents and polymers commonly used in bioconjugation applications.

Why choose Fortis?

We are dedicated to providing superior products, as well as offering the support our customers need to be successful with particle integration.

Do you perform custom conjugations or assay development/consulting?

Yes! Please visit us at fortislife.com/custom-conjugation-services-for-lateral-flow to learn more about our custom conjugation services.

Conclusions

The optimization of an LFA test is a challenging, multi-step process. We hope that this guide has provided some insight into the necessary steps and provided ideas on how to improve your lateral flow assay . We also recognize that there are many research scientists and developers who have additional knowledge on the fabrication and optimization of LFA and we'd love to hear from you so we can incorporate your knowledge in this document. With the advent of novel probes that significantly improve sensitivity, advancement in multiplexed formats, and the increasing adoption of lateral flow assays in emerging fields such as oncology, lateral flow assays are currently experiencing a period of explosive growth and innovation.

We're here to help in any way that we can – for technical assistance, information on custom capabilities, or collaboration efforts, please contact us at (858) 565-4227 x2 or email us at info@nanocomposix.com.

For more information, visit fortislife.com/lateral-flow