Lateral Flow Assay Development Guide

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This document provides instructions and protocols to help our customers maximize the sensitivity and performance of lateral flow assays when using nanoComposix nanoparticle probes. We provide a step-by-step walkthrough of all stages of lateral flow assay design, best-mode protocols including specifics on material selection, and 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 contact us at *info@nanocomposix.com* or (858) 565-4227.

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Introduction to Lateral Flow

Overview

Lateral flow assays (LFAs) are rapid and inexpensive diagnostic devices that can be used to test for a target substance (analyte) in a sample. Some of the advantages to the LFA format are that these assays have a long shelf life, do not require refrigerated storage, and a result can be obtained in a short time without additional processing, external equipment, or extensive training. The simplicity of this diagnostic is especially important for point-of-care or field-based diagnostics.

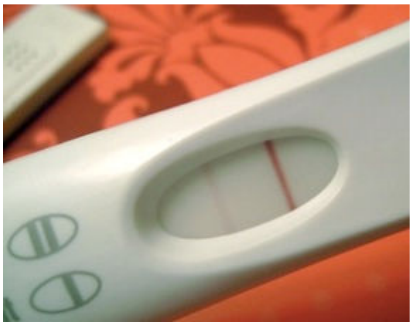

Figure 1: A commercial pregnancy test which uses 40 nm gold nanoparticles as the detection probe. The faint red line indicates that the subject is pregnant. The darker red line indicates that the test was valid.

Because of their low cost, billions of tests are produced each year to test for a variety of different analytes. The most common and well known example of a LFA is an over-the-counter pregnancy test. In this configuration, a colored line at the test location indicates a positive test, and a second line at the control location indicates that the test was valid (**Figure 1**). The colored line(s) that appear after the addition of a sample are the result of many targeted (conjugated) plasmonic nanoparticles being captured by the antibody or capture reagent immobilized at the test and/or control lines.

Colloidal 40 nm gold nanoparticles are the industry standard for the red colored line that indicates if the test is negative or positive. The red color arises from an optical plasmon resonance of the 40 nm gold nanoparticles, which strongly absorb green and blue light. While 40 nm gold nanoparticles have found widespread use, nanoComposix has developed a variety of high-quality plasmonic nanoparticles with various optical signatures including 40 nm gold and other unique nanoparticles that give rise to increased sensitivity in lateral flow assays (**Figure 2**). Not only will these new probes help to develop lateral flow tests with better sensitivity and reproducibility, the results from these ultra-sensitive diagnostics can be read by eye, or quantified with inexpensive digital readers (e.g. cell phone or in-cassette readers). We believe this combination of better probes and disposable readers has the potential to revolutionize the point of care diagnostic industry.

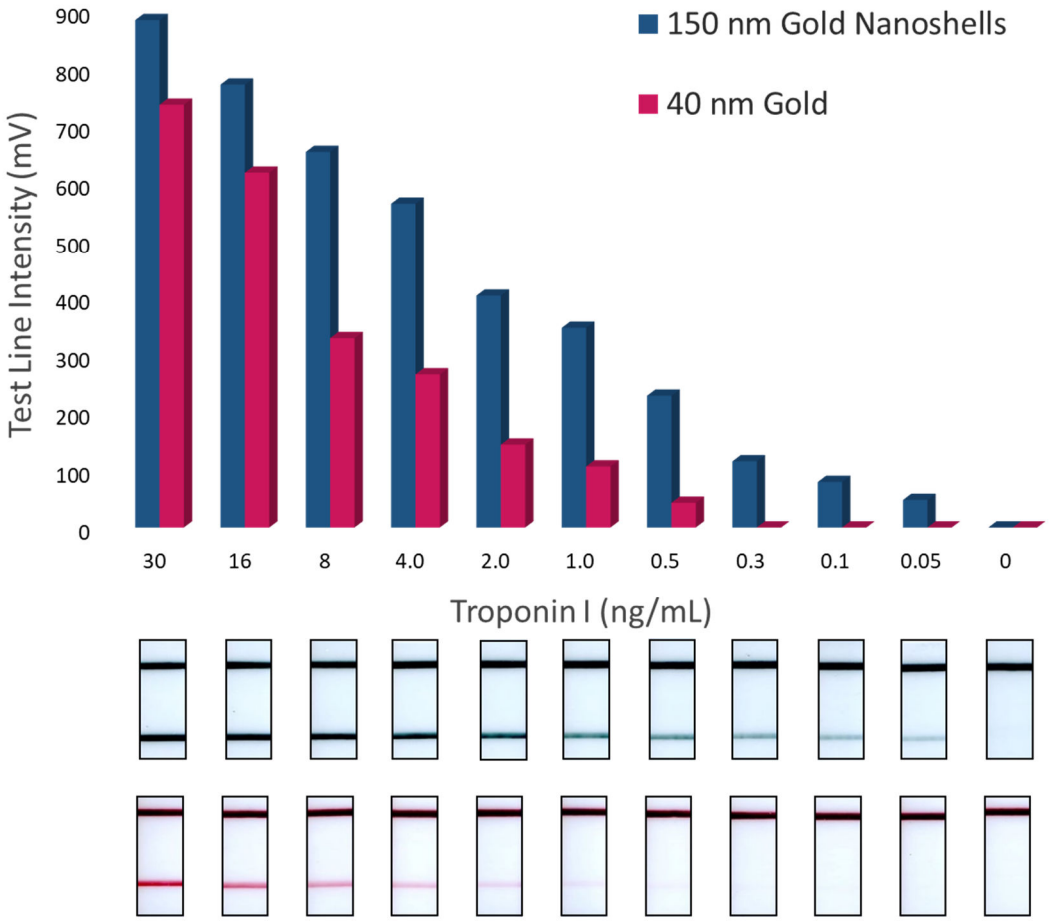

Figure 2: Lateral flow assay with serial dilutions of troponin I showing the **10X** increase in sensitivity when using gold nanoshells (blue) as the probe over 40 nm spherical gold (red). Test line intensity measured using the Qiagen ESEQuant benchtop reader.

Traditional Lateral Flow Components

The lateral flow assay contains several key components:

- Sample pad: *Provides sample absorption, and controls distribution and flow of sample onto the conjugate pad*
- Conjugate pad: *A medium for dispensing and drying nanoparticle-antibody conjugates, aids in controlled release of re-solubilized conjugate onto the nitrocellulose membrane*
- Nitrocellulose membrane: *Provides the ideal solid phase for imobilizing test and control line reagents*

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

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 of 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 (2-20 minutes) indicates the presence or absence of the target analyte.

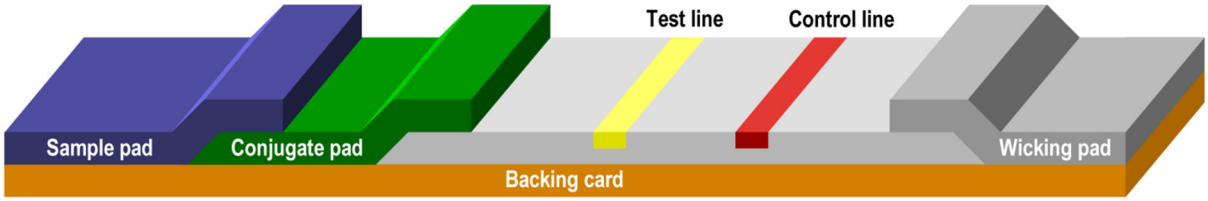

Figure 3: Schematic of a generic lateral flow test. The **sample pad** absorbs the sample and transports the sample to the conjugate pad. The **conjugate pad** contains the dried down antibody-nanoparticle conjugate. The nitrocellulose membrane has test and control lines that show the assay results.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

At the core of a lateral flow assay are the "conjugates" which are also called detector nanoparticles or probes. These are brightly colored nanoparticles are functionalized with proteins, most ofen 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. The test line contains an immobilized protein that either binds to the target analyte or competes with the target analyte for binding to show the 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 species-specific anti-IgG. The sample to be analyzed (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 liquid 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) can detect a wide range of targets, and can be configured in a variety of formats. 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 are "sandwich" and "competitive" assays, which 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 where 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 steroids and drugs of abuse. In a competitive assay, the test line contains the target analyte molecule (usually a protein-analyte complex). The nanoparticles are conjugated to an antibody that recognizes the 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.

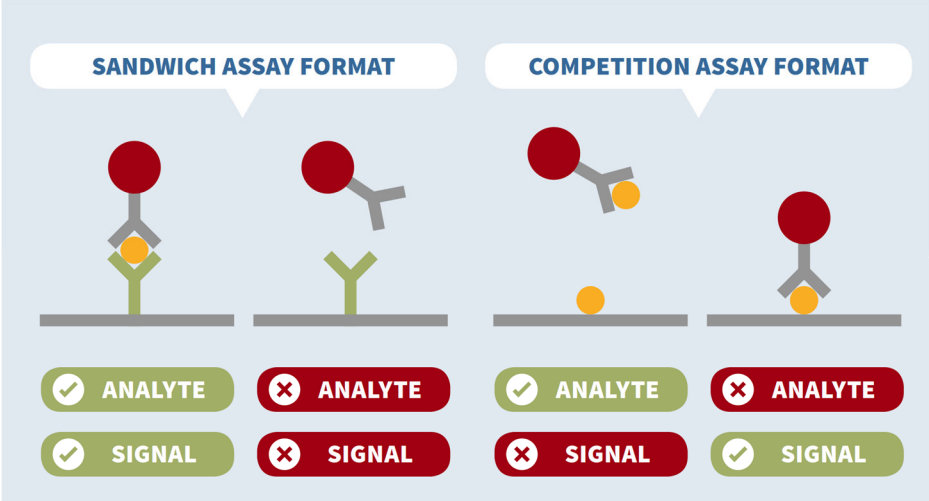

Figure 4: Schematic of sandwich assay format and competitive assay format.

Both assay formats typically have a second "control" line immobilized on the nitrocellulose membrane to allow the user to verify that the assay has not been compromised and the result is valid. The control line is typically a species-specific anti-IgG, and will bind to the antibody that is conjugated to the nanoparticle probe. For both assay types the control line should be visible whether or not the analyte is present in the sample. Typically 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, such as those that detect pregnancy, a simple "yes" or "no" is all that is necessary. 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. Recent 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**).

Nanoparticles

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

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

The methods and techniques used to conjugate antibodies to the surface of nanoparticles are critical for optimizing the performance of lateral flow assays. For gold nanoparticles, antibodies can either be physisorbed to the surface, also referred to as passive adsorption, 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 is 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 attractions between the antibody 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-gold ratio can 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 in order 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 optimimization. Secondly, some antibodies may detach from the nanoparticle surface under certain 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 chemistries. For our BioReady products that are optimized for lateral flow, we typically 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. NanoComposix 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

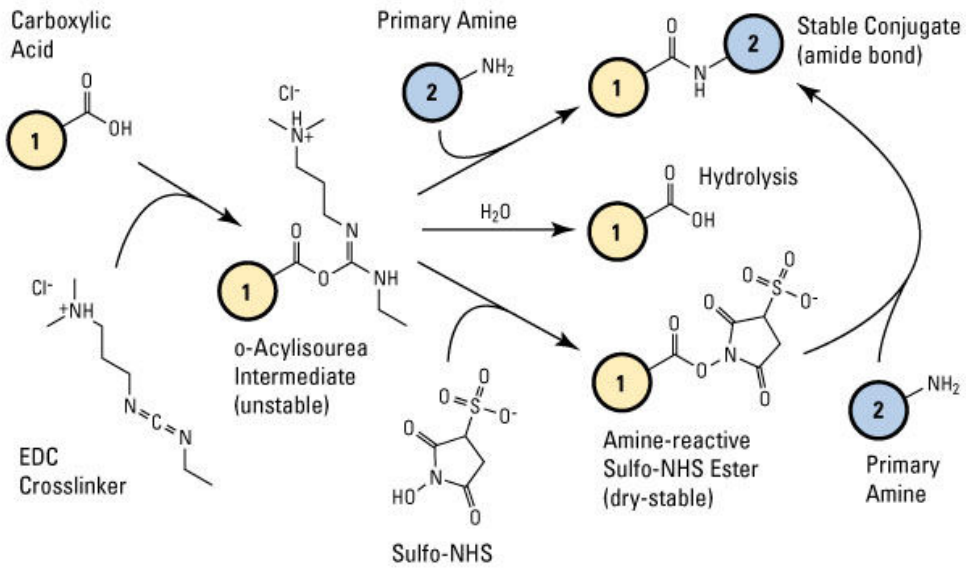

Figure 6: EDC-NHS reaction scheme – from Thermo-Fisher.

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 beginning the development process for a lateral flow assay, there are a number of different 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 requirement decisions are drivin by clinical relevance. It is important to consider all design inputs that are required prior to starting assay development.

A critical first step is to identify a control assay if possible, whether in lateral flow or on a different platform, that can independently measure the analyte of interest and to validate the assay if your assay 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 a 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 nanoComposix, we have experience with assays in both competitive and sandwich format and in

qualitative and quantitative platforms. For information regarding our custom development abilities, contact *info@nanocomposix.com*.

Lateral Flow Assay Design

An overview of a typical small-scale LFA manufacturing process at nanoComposix is shown in **Figure 7**. 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.

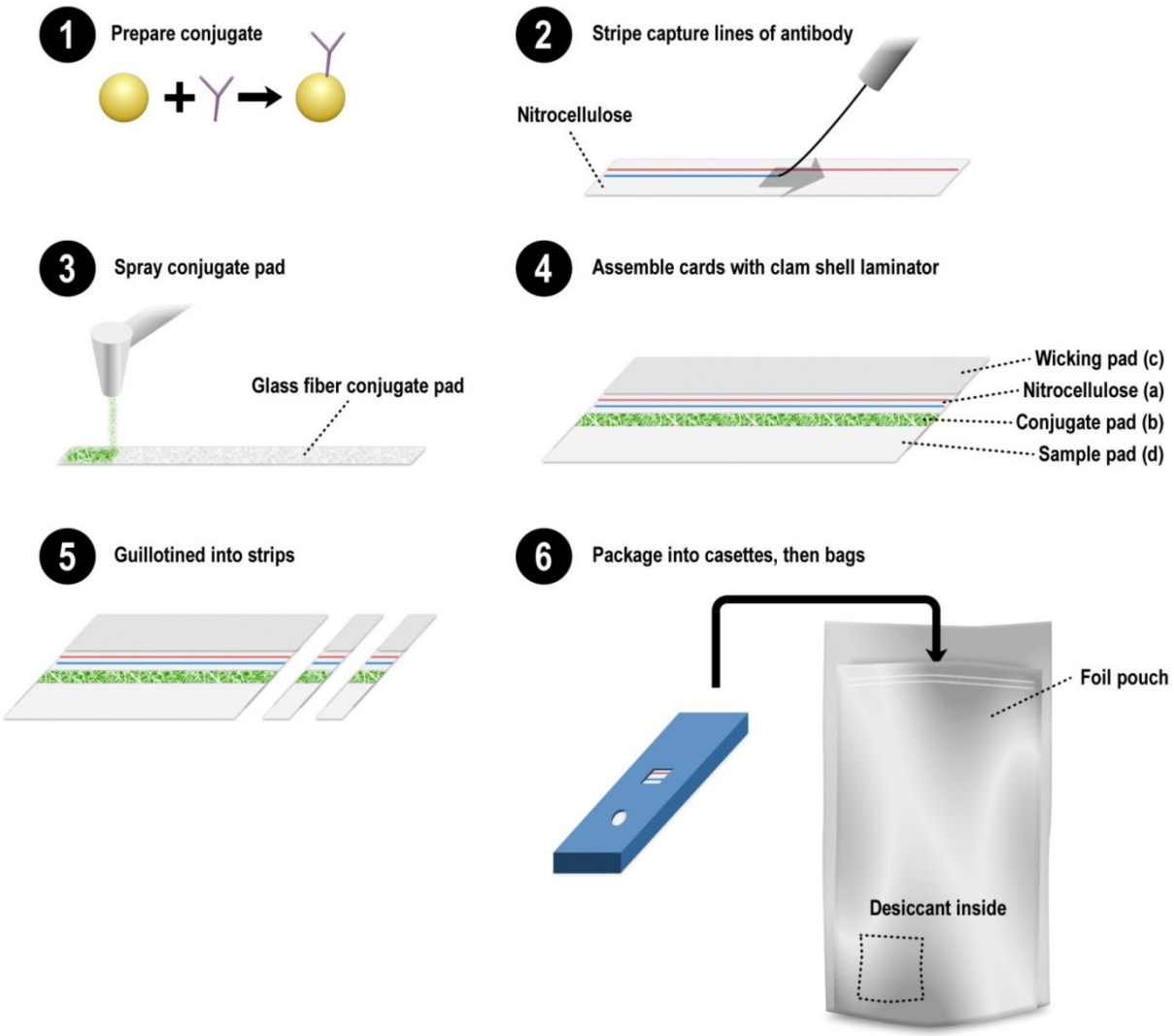

Figure 7: General procedure for the card-based assembly of a lateral flow device consisting of (1) conjugate preparation, (2) striping of capture lines, (3) spraying conjugate pad, (4) assembly of cards, (5) strip cutting, and (6) packaging into cassettes.

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 7, 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 7, Step 2**). The conjugate is applied to the glass fiber conjugate pad using a non-contact spray head on the dispenser (**Figure 7, 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 clam shell laminator. The laminator ensures an accurate placement of components onto the adhesive backing card with the correct overlaps and controlled application pressure. These fully laminated backing cards are refered to as "master cards". To assemble a master card, the nitrocellulose is applied first, followed by the conjugate pad, wick pad, and then sample pad (**Figure 7, Step 4**). The assembled cards are then cut into individual strips with an automated guillotine (**Figure 7, Step 5**), and assembled into a plastic cassette that is sealed in a foil pouch with desiccant (**Figure 7, 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, there are many optimization steps that 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 step-wise process. Most of these steps need to happen concurrently and will need to be re-visited multiple times throughout the development cycle.

Step #1: Nanoparticle Selection

NanoComposix has a line of BioReady products that is specifically tailored for antibody conjugation. We offer protocols and technical support for conjugation to each particle type. The following sections list the benefits and trade-offs of the different particle sizes, shapes and surfaces:

BIOREADY 40 NM BARE GOLD (CARBONATE OR CITRATE SURFACE)

Our BioReady 40 nm bare gold nanoparticles have a "naked" particle surface with only a weakly associated carbonate or citrate molecule to stabilize the particle, and can be conjugated to proteins through passive adsorption (also referred to as physisorption). The most common buffer for bare nanoparticles is trisodium citrate, which is used as a reductant in many gold nanoparticle

fabrication methods and provides a balance between stability during particle formation and displaceability when making particle conjugates. Each of the three carboxylic acids weakly bind to the particle surface but are readily displaced in the presence of a protein. We also offer the "naked" surface in a carbonate buffer, which is a smaller and less complex molecule with a lower affinity to the gold nanoparticle surface than citrate. The greater displaceability of the carbonate molecules may produce better performing conjugates.

Both of the 40nm bare gold nanoparticles with either the carbonate or citrate surface can be used for passive adsorption to proteins. The mechanism of adsorption is based on van der Waals interactions between the proteins (e.g. antibodies) and the surface of the particles. The resulting forces between the antibody and the nanoparticle are influenced by the coupling environment. The BioReady 40 nm carbonate gold is provided at an optical density (OD) of 20 at pH 5.5-6, and the 40 nm citrate gold is provided at an OD of 20 at pH 6.5-7. A pH titration should be performed to optimize the conjugation efficacy.

Advantages:

- Traditional method of conjugate preparation
- Very little chemistry involved
- Highly reproducible nanoparticle synthesis method with low batch-to-batch variance which is critical for semi-quantitative and quantitative assay development
- Low cost

Disadvantages:

- pH sweep required for adsorption optimization
- Whole antibodies or thiolated ligands required
- Proteins are not covalently attached to particle surface and can desorb
- Risk of aggregation if conditions are not optimized
- Binding mechanism is antibody dependent
- Need to carry out multiple trial conjugations

BIOREADY 40 NM CARBOXYL GOLD

NanoComposix BioReady 40 nm carboxyl (-COOH) gold is an effective and economical nanoparticle for covalent conjugations to proteins through carbodiimide crosslinker chemistry. Covalent coupling of proteins (e.g. antibodies) to a gold nanoparticle surface yields robust and stable gold particle conjugates. The nanoparticles are surface functionalized with a tightly bound

monolayer that contains terminal carboxylic acid functional groups which can be activated through EDC/Sulfo-NHS chemistry to generate gold nanoparticle-antibody amide bonds.

Advantages:

- Whole antibodies, antibody fragments, and small molecules can be irreversibly bound
- Generally less antibody is required than for passive adsorption
- Stable and irreversible amide bond formed
- Improved control over antibody/particle loading (difficult to accomplish in passive adsorption because of colloidal stability challenges)
- More stable compared to passive conjugates in a challenging sample matrices and stable in a wider range of pH and high surfactant and detergent loading

Disadvantages:

- Requires additional step to activate the -COOH surface with EDC/Sulfo-NHS chemistry
- Fewer antibodies on the surface compared with passive coupling methods for an equivilant particle size

BIOREADY 40 NM NHS GOLD

NanoComposix BioReady 40 nm NHS gold can be covalently conjugated to primary amines (-NH_2) of proteins in a simplified procedure. Covalent coupling of proteins (e.g. antibodies) to a gold nanoparticle surface yields robust and reliable gold particle conjugates. The BioReady 40 nm NHS gold nanoparticles are surface functionalized with an active NHS ester to generate gold nanoparticle-antibody amide bonds, eliminating the need for the user to perform the intermediary EDC/Sulfo-NHS chemistry steps. The particles are supplied as a lyophilized powder that can be resuspended with a buffer to covalently bind to an added antibody. This coupling reaction is rapid, simple, robust, and requires little optimization.

Advantages:

- Fast– stable gold conjugates in as little as 15 minutes hands-on-time
- Convenient–rapidly screen multiple antibodies for assay development without having to perform pH or salt optimizations for each antibody
- Economical- reduced antibody loading and minimal pH optimization required

Disadvantages:

- NHS gold solution must be used immediately upon resuspension

- At larger scales, it is much more cost effective to perform EDC/NHS chemistry with nanoparticles having carboxylic acid surfaces and performing EDC/NHS chemistry immediately before antibody binding may increase assay sensitivity.
- Moderately lower binding efficiency compared to carboxylic acid surface due to the inherent half-life of the NHS ester intermediate

BIOREADY 150 NM CARBOXYL GOLD NANOSHELLS FOR INCREASED SENSITIVITY

At nanoComposix we fabricate hundreds of different sizes and shapes of metal nanoparticles that strongly interact with light due to their plasmon resonance. While 40 nm gold has historically been the nanoparticle of choice for lateral flow assays, gold nanoshells, another type of plasmonic nanoparticle, can dramatically increase the sensitivity of lateral flow assays because each particle is 30x more strongly colored compared to 40 nm gold (**Figure 8**). Because of the dramatic increase in color displayed by the 150 nm gold nanoshells versus the 40 nm gold particles, fewer binding events are required in order to see a result at the test line in a lateral flow assay. The gold nanoshells consist of a 120 nm silica core surrounded by a thin 15 nm shell of gold. The gold nanoshells have a much larger diameter than 40 nm gold nanoparticles but flow unimpeded through the nitrocellulose membrane because of the low-density silica core. The 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. Due to the larger particle size, covalent binding chemistry is used to link antibodies to the surface of nanoshells.

Advantages:

- Whole antibodies, antibody fragments, and small molecules can be irreversibly bound
- Generally less antibody is required than for passive adsorption
- Stable and irreversible amide bond formed
- Improved control over antibody/particle loading (difficult to accomplish in passive adsorption because of colloidal stability challenges)
- Up to 20X increase in lateral flow sensitivity
- NHS and COOH based covalent linkage chemistry available
- More stable compared to passive conjugates in a challenging sample matrices and stable in a wider range of pH and high surfactant and detergent loading

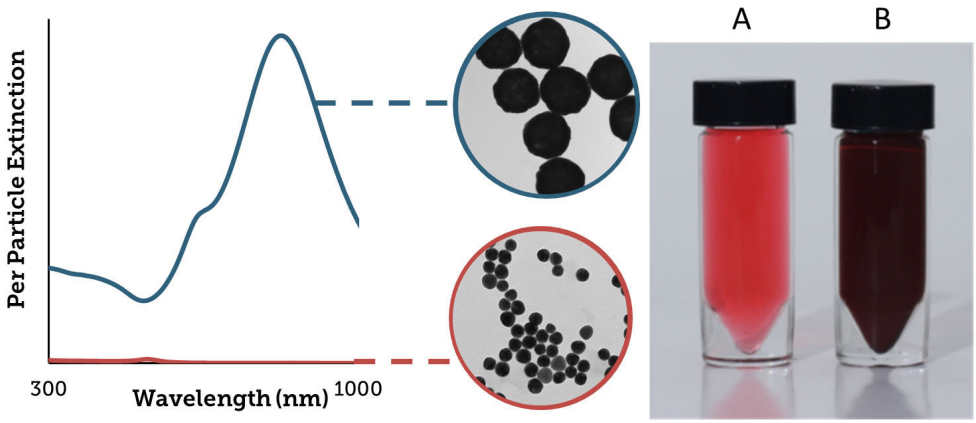

Figure 8: The graph shows150 nm BioReady gold nanoshells absorb light with a per particle extinction coefficient that is 35-fold larger than 40 nm gold spherical nanoparticles. The vial of 40 nm gold nanopartcles (A) and the vial of gold nanoshells (B) are at the same particle number concentration and highlight the difference in color intensity.

Disadvantages:

- Requires additional optimization when switching from 40 nm gold nanoparticles
- Since the extinction of a nanoshell is much larger than a gold nanoparticle, there are fewer particles per OD when purchased in solution. When optimized, a higher OD of particles may be necessary on each strip in order to maximize sensitivity.

OTHER PROBES FOR LATERAL FLOW

There are a number of other probes used in lateral flow assays that are not gold. Dyed polystyrene particles (typically 200 nm or greater in size) and cellulose beads can be used for increasing visible signatures on strips. Cellulose beads (e.g. Asahi Kasei Fibers Corporation) have large diameters and work well for certain systems but can also have stability issues in certain matrices and be challenging to optimize. For higher sensitivity, fluorescent probes can be used and can provide 10 – 100X increases in sensitivity over 40 nm gold. However, these require a specialized fluorescent reader to analyze and quantify the result. Europium beads and up-converting nanoparticles are two fluorescent particles that are commonly used in fluorescent LFA assays.

Selection of the nanoparticle probe will be based on the type of assay, sensitivity requirements, price-point requrements, and the available reader technology. This is an important decision since many of the subsequent steps in the lateral flow development process will require optimization that is dependent on which nanoparticle was used as a probe. For help determining which probe is best suited for your application, please contact us at *info@nanocomposix.com*.

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 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 is needed.

ANTIBODY SELECTION

Whether you are buying 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 precess is to research what antibodies are available for your specific application. The number of antibodies available for a specific analyte varies greatly. If antibodies are commercially available, aim at acquiring and testing as many as possible to perform 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 or specifc amio-acid sequences. Immunization of rabbit, mouse, or other host species typically yields multiple unique antibody clones whichever immunization antigen you select. Custom manufacturing does not necessarily guarantee success of finding a clinically relevant antibody, but it does provide some valuable benefits later on in development as well. Mainly, control of antibody supply to mitigate manufacturing risk, as well 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 persuing 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 lower-risk for manufacturing. In some cases, polyclonal antibodies may provide the best sensitivity due in part to the their ability to bind multiple portions of the analyte.

Monoclonal antibodies are assigned clone number, 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 of 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.

Table 1: Antibody pair evaluation matrix

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

When choosing an appropriate antibody pair for a sandwich assay, we test every possible combination of 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 conjugated prepared 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.

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

	POLYCLONAL	MONOCLONAL
ADVANTAGES	• Inexpensive to produce • High affinity • Recognize multiple epitopes (generally provides more robust detection) • Polyclonal antibodies are often preferred for 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 of 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)

The advantage and disadvantages of polyclonal vs. monoclonal antibodies are listed in **Table 2**. Monoclonal antibodies are antibodies that have been grown from a single cloned hybridoma, are structurally identical, and recognize a single epitope on an antigen. Polyclonal antibodies are a heterogeneous mixture of antibodies that potentially recognize multiple epitopes on an antigen. 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 the lateral flow development, empirical testing is the best way to determine the optimal conditions for each assay.

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 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 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 control. The line will be visible in the presence or absence of 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 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 provies the readout of the 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 AND 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. In 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) run more slowly than non-viscous samples such as urine and may flow better using faster membranes.

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

RELATIVE FLOW TIME	RELATIVE PORE SIZE	RELATIVE SENSITIVITY	EXAMPLES
FAST	LARGE	LOW	Millipore: HF 75, 90 Sartorius: CN 95 MDI: NC 15 µm Whatman/GE: AE 98, AE99
MEDIUM	MEDIUM	MEDIUM	Millipore: HF 120, 135 Satorius: CN 140, CN 150 MDI: NC 8 µm Whatman/GE: FF120 HP
SLOW	SMALL	HIGH	Millipore: HF 180 MDI: NC 5 µm Whatman/GE: FF170HP

Membranes are available from a number of manufacturers including MDI, EMD Millipore, Whatman/GE, and Sartorius. Manufacturers treat their membranes with proprietary mixtures of surfactants and other chemicals to make them hydrophilic. 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 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 peforms similarly between lots.

MEMBRANE STRIPING

Another step in the lateral flow assay design process is to stripe the test and control antibody lines onto the nitrocellulose membranes. At nanoComposix, 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-contact dispensing. The nitrocellulose membranes should not be stored in a desiccated environment prior to striping, but rather 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 are too damp will result in a widened test line that may decrease the signal intensity. Always refer to the storage and handling guidelines provided by the manufacturer. Overnight acclimation of the nitrocellulose membrane in a humidity controlled environment is a standard practice to ensure the membrane will bee striped consistently. Once the nitrocellulose has been striped and dried, it is important that the humidity 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.

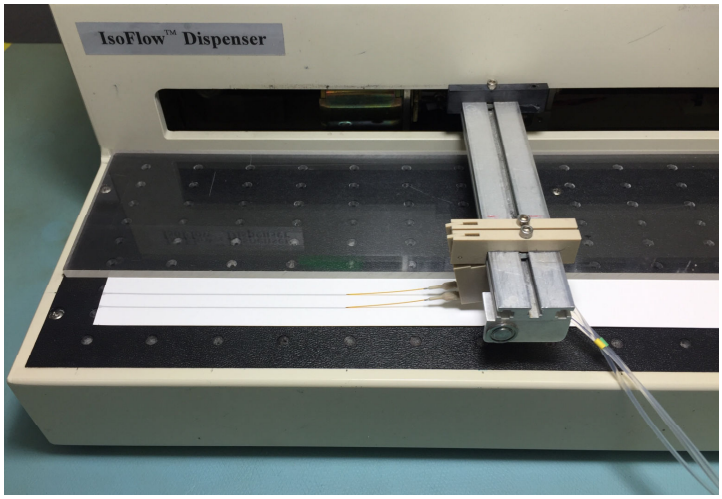

Figure 9: Striping the nitrocellulose membrane with test and control solutions using an Imagene Isoflow dispenser.

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). 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 dispense speed typically used for the Isoflow dispenser at nanoComposix 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. The antibodies used for striping do not need to be purified from preservatives, and can be diluted in 1X PBS buffer.

For any lateral flow assay, the buffer in which a particluar protein is striped can have pronounced effects on the end results. 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 charactersitics.

After striping the membranes, it is important to mark each membrane with the line location and orientation of the test and control line. 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 recomenned that membranes be cured in in a forced-air

convection oven. The curing, and subsequent over-night 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 °C in a forced-air convection oven, followed by overnight storage in a desiccated environment. Final packaging should also include dessicant pouches to ensure the nitrocellulose membrane is not exposed to any moisture. Optimizing the membrane handling post striping will be important to developing 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. Blocking buffers can include sugars, polymers, proteins, and/or surfactants. While some developers may utilize this step, it can be time consuming during the optimization process and add uneccesary steps when manufacturing at scale. Most nitrocellulose mebranes are treated by the manufacture with a proprietary solution in order to make the membranes hydrophylic. Blocking the membrane may wash these reagents off, so any membrane treatment most be carefully evaluated. Alternatively, the chemicals utilized to enhance performance may be incorporated in 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, nanoComposix offers 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, 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. At nanoComposix, we have extensive experience in bio-conjugation to nanoparticles. We are able to 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 AND 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 particles 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** below, 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 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.

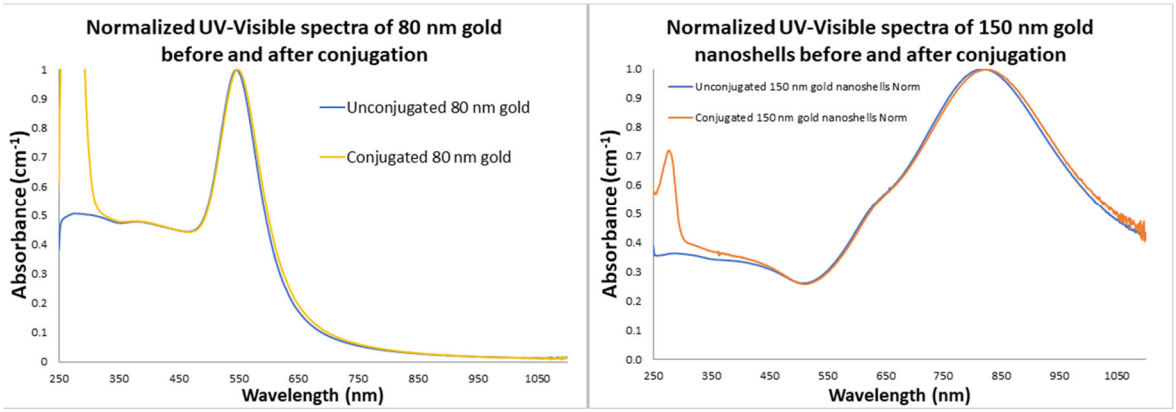

Figure 10: (Left) Normalized UV-vis spectra of 80 nm gold before and after successful conjugation, corrected by dividing the entire spectra λmax, shows any changes in shape of the UV-visible spectra. (Right) Normalized UV-vis spectra of 150 nm gold nanoshells before and after successful conjugation.

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 remaining 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 peaks to red-shift to longer wavelengths.

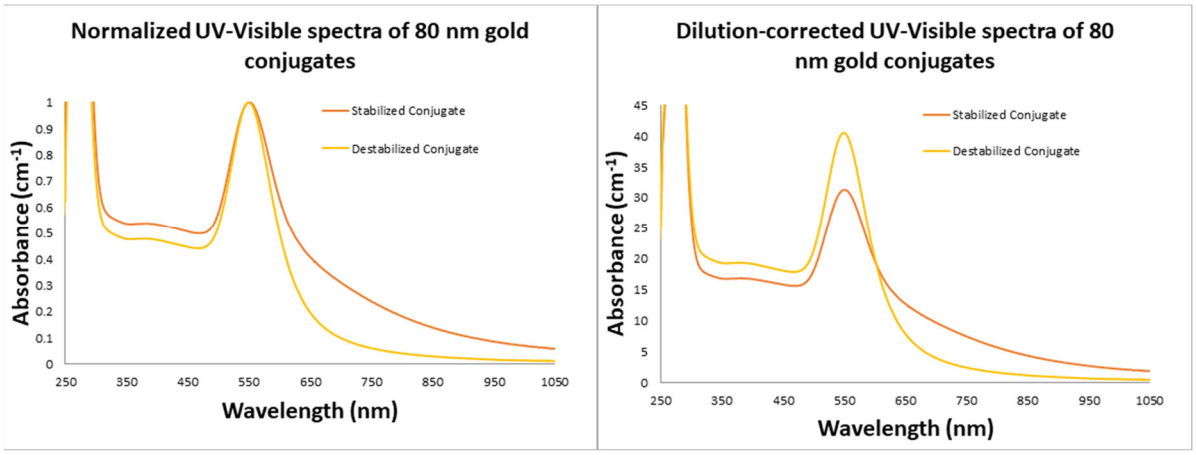

Figure 11: (Left) Normalized UV-vis spectra, corrected by dividing the entire spectra λmax, shows any changes in shape of the UV-visible spectra. (Right) Dilution-corrected UV-vis spectra helps to look for changes in the absorbance or optical density. UV-vis spectra of a colloidally stable 80 nm gold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

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 λmax) helps to observe any changes in shape of the UV-visible spectra. A dilution-corrected UV-vis spectra allows us to look for changes in the absorbance or optical density. **Figure 12** demonstrates the same observations but with 150 nm gold nanoshell conjugates.

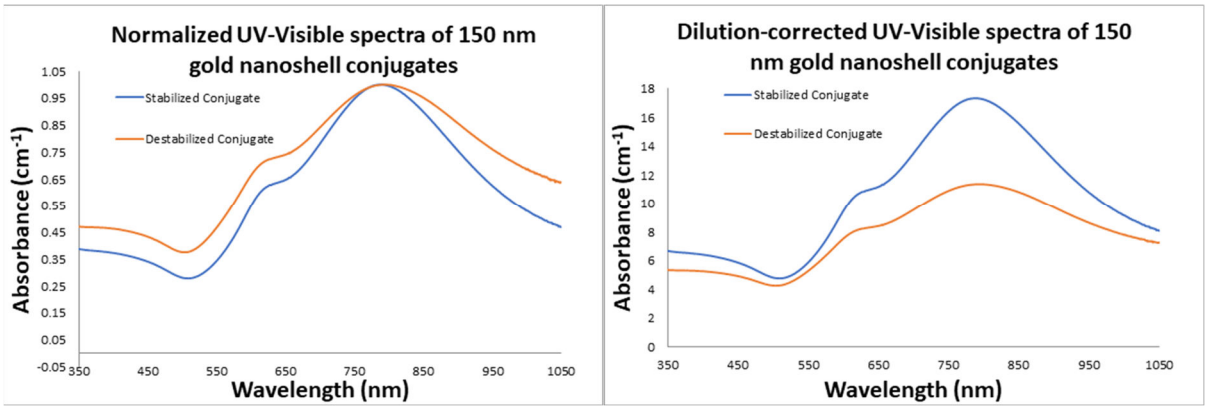

Figure 12: UV-vis spectra of a colloidally stable 150 nm gold nanoshell conjugate and a 150 nm gold nanoshell conjugate with poor colloidal stability. (Left) normalized UV-vis spectra, corrected by dividing the entire spectra λmax, shows any changes in shape of the UV-visible spectra. (Right) Dilution-corrected 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 μL/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 antibodies 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 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 protein binding sites on the membrane prior to the conjugate interaction. This reduces non-specific interactions. Adding blocking reagents and non-ionic surfactants to the conjugate 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

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 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 should be evaluated to ensure that the sample has the optimal flow and treatment before the sample reaches the reagents in the system.

Sample pads can be made from various materials such as glass fiber, cellulose, cotton, and synthetic material. The absorption capacity is an important product specification as it can dictate the sample volume that will be allowed per test. The sample medium 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 composition 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 mediums, such as urine and saliva, the composition of the sample can vary significantly depending on the individual, time of day, food and drink consumed before sample collection, as well as many other biological factors. Treating the sample pad with an optimized buffer can aid in "normalizing" the samples before they reach the conjugate to prevent any negative interactions that may occur from the differences in pH, protein composition, mucins, salt concentrations, and any molecules that may cause non-specific interactions with the antibody

system. In other sample mediums, such as whole blood and solubilized solids, it is critical to prevent the passage of unwanted material. For these samples, the sample pad can act as a filter. The sample pad will retain the unwanted particulates while allowing the fluid containing the analyte of interest to flow through the test strip. In the case of whole blood, a filter can be used that will hold back the red blood cells while allowing the plasma/serum to pass through the assay (**Figure 13**). It is also critical to understand whether the sample pad material has an affinity for the analyte of interest.

Treating the sample pad with an optimized buffer can enhance assay performance by mitigatating 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 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 these lysed cells through the membrane.

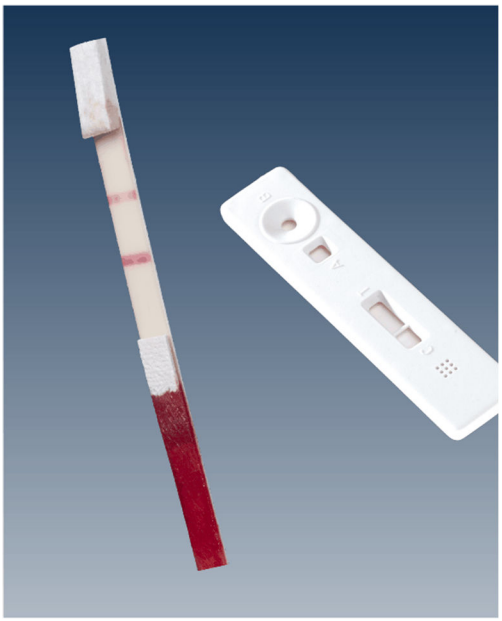

Figure 13: Lateral flow assay with sample pad that has a red blood cell filter. Image from mdimembrane.com

Sample pad treatment can be performed by immersion, 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°C for 1-2 hours and then cured overnight and stored in a desiccated environment (<20% relative humidity) at 18-25°C.

Step #6: Absorbent Pad (Wick) Selection

The wick pad needs to absorb all of the reagents that were not taken up by the test and control lines, while maintaining capillary flow through the membrane to clear the background. It also prevents backflow of the excess reagents for as long as possible, at a minimum, past the read time of the test. The material and size of wick pad should be selected such that the absorption capacity is much higher than the sample and running buffer volume. Absorbent pad materials can be purchased from companies such as Millipore, Whatman/GE, or Ahlstrom. It is important to note that different materials should be screened for best results. A thicker material does not necessarily perform better or have a higher absorption capacity than a thinner material.

Step #7: Test Strip Assembly

At larger scale manufacturing (e.g. >1 million tests per year), reel to reel systems can be employed to facilitate production, but card systems are more simple and economical route for smaller scale manufacturing.

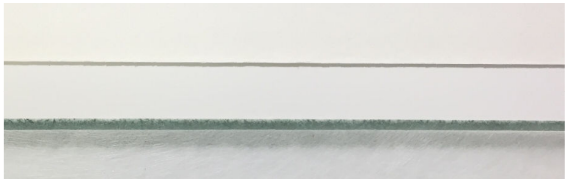

Figure 14: Assembled 30 cm master card with 150 nm gold nanoshell conjugate. This card is subsequently cut into the final test strips orthogonal to its length.

NanoComposix uses 30 cm backing cards from Lohmann for assembling the lateral flow tests (**Figure 14**). These cards have a LFA-compatible adhesive coating underneath a series of peel off layers, which are removed to attach the various components of the test strips (**Figure 7 (4)**). 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 variability of component placement, in turn generating more reproducible test results with lower variation.

Once a master card is assembled, the next step is to cut the card into strips. An automated guillotine (e.g. Kinematic, BioDot) should be used to accurately cut strips with a high degree of reproducibility. Strip widths may vary between 3 mm and 6 mm, depending on the specific assay requirements. Thinner strips are more cost effective (higher number of strips per card) but can be less accurate due to edge effects. Quantitative assays are most commonly cut to 5-6 mm widths.

The cassette that houses the test strip can be one of the most critical components to achieve a reproducible and reliable assay, which is especially important for quantitative tests. The cassette provides optimal flow control by applying pressure at appropriate points on the strip to ensure 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 is customized to the lengths, widths, and thicknesses of each component. Control over the pressure can control the flow rate of the sample fluid, allowing for longer or shorter incubation times of conjugate with sample analyte. For large scale production, a custom designed cassette from an experienced industrial design company is necessary, preferably a company who already has lateral flow cassette expertise. When using a custom design, it is always important to keep in mind the mitigation of manufacturing risk down stream. This is an often overlooked, but critical component of the designing process. For initial testing, existing generic cassettes may be sufficient.

For some assays, a quantitative read-out is not required and a dipstick format may be sufficient in running the strip. In this format, the strip is not placed inside a plastic cassette. Cover tape may be used to provide some flow control and to hold all of the components of the strip together.

Step #8: Running the Assay

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

DIPSTICK ASSAY (LIQUID CONJUGATE)

For 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 or 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 together in a well of a 96 well plate, or in a small test tube (**Figure 15**). The strip is then dipped in the mixture and the solution 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.

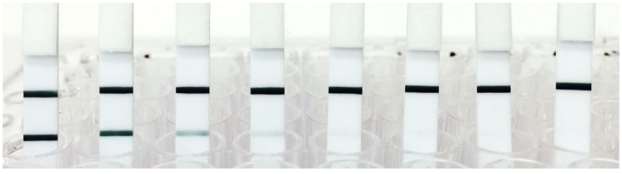

Figure 15: Running liquid conjugate in half strip format.

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 material used as the sample and conjugate pad, and the sample medium being tested.

During assay development, initial screening should be performed in a "clean" system. A clean system refers to a purified analyte (i.e. protein or small molecule) spiked into a buffer at known concentrations. A clean system minimizes the matrix dependent effects of the matrix you are examining (e.g. urine, plasma, saliva, etc), and provides a less variable system for running direct comparisons such as antibody pair evaluation, nanoparticle selection, test strip materials, and buffers.

Once the assay is partially optimized in a clean system, it is important to move into a system that more closely resembles the real clinical samples, or to use the clinical samples if they can be obtained and validated. One option is to transfer into a system that uses purified analyte spiked into artificial samples, such as artificial saliva or artificial urine which can be purchased commercially. Although these artificial samples may mimic certain aspects of the real samples such as pH and viscosity, the chemical composition will vary. For example, artificial saliva can mimic the pH and viscosity of a real saliva sample, but the proteins and mucins that are present in real saliva may not be present in the artificial samples. Thus, when switching to the real samples, the results may be affected by the other components present. Another option is to switch to purified analyte spiked at known concentrations into the real sample medium that has little or no endogenous analyte present. This may be possible when detecting biomarkers that are not normally present in samples from healthy patients. Finally, it is important to switch to real samples with endogenous analyte as soon as availability allows.

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-specficic 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 that you have an objective means of quantifying the output of the test strips. 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.

A 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. At nanoComposix we use gradient score cards where the strength of the lateral flow line can be measured against a printed line intensity in order give a semi-quantitative score, this is useful for ealy 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 nanoComposix we use a Lumos camera-based reader, or Qiagen reader that provides a quantitative readout in approximately 30 seconds. We also have a number of cell-phone based reader technologies in development.

Step #10: Assay Optimization

There are many components that need to be meticulously optimized to develop a high sensitivity lateral flow assay. 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. For quantitative assays, the standardization analyte and matrices will also have to be optimized. There is a relationship among all of these components that needs to be carefully balanced to produce an effective and functional assay. Accordingly, the development process is not linear. After each stage of optimization, the preceding stages often need to be revisited and re-optimized, resulting in an iterative and recursive process **(Figure 16).** When optimizing an assay, two critical metrics to evaluate are increased signal intensity and elimination of non-specific signals. Because each antibody and conjugate is different, it is important to re-screen all parameters individually for each antibody.

As a general 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 may seem trivial but could significantly affect the assay results down stream.

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.

When optimizing a lateral flow assay, many issues may be encountered such as the instability of conjugates, aggregation at the interface of the conjugate pad and membrane, non-specific binding, or lack of desired sensitivity. All of the steps listed above may be re-visited to address some of these issues, such as sample pad treatment, conjugate pad treatment, membrane selection, and running buffer.

Aggregation at the interface of the conjugate pad and membrane, flocculation of the particles to the side of the tube, non-specific binding at the test line, or a change in UV-Vis measurements of the conjugate are all indications that the conjugate is unstable. We provide protocols and technical support for conjugating to our BioReady particles and many of the optimizations that we perform at nanoComposix to stabilize and improve the efficacy of the conjugate are outlined below.

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 plasticizer 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 lo-bind tubes are desireable, we roccomend performing the activation step in lobcon tubes, and after the activated particles have been centrifuged and resuspended in reaction buffer, the particles may be transferred to a lo-bind tube prior to the addition of 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 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 5mM potassium phosphate, 5 mg/mL PEG20 at pH 7.4. While activation of NHS esters is most efficient at ph 5, 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 μg antibody per 1 mL OD 20 particles and our 40 nm carboxyl gold with an antibody ratio of 2-60 μg 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 0.5 XPBS, 0.5% BSA, 1% Tween20, 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. 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 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 exchanges (link to antibody purification protocol).

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 in order 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 CARBONATE 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. 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 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 30X less 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 the 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 a number of 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 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 than 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 (http://scantibodies.com/hbr/), 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 DECREASE OF TEST AND CONTROL LINE WHEN SWITCHING FROM "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 in 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 has been stored properly and that it is prepared just prior to conjugation. EDC should always be stored at -20°C and Sulfo-NHS between 4-8°C. 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 colloid solution.

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 NANOCOMPOSIX?

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 contact us regarding our custom capabilities and collaboration efforts.

Conclusions

Optimization of a LFA test to maximize sensitivity 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 diagnostic. We also recognize that there are many research scientists who have additional knowledge on the fabrication and optimization of LFA and we'd love to hear from you so we could add your knowledge to this document. It is our belief that new probes that increase the sensitivity of LFA coupled with the development of inexpensive quantitative smart-phone based readers, will enable a second wave of new applications and markets.

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