nanoComposix

Lateral Flow Assay Development Guide

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

Contents

Lateral Flow Assay Development Guide 1

Introduction to Lateral Flow 3

- OVERVIEW 3
- LATERAL FLOW COMPONENTS 4
- ASSAY FORMATS 6
- NANOPARTICLES 7

Preliminary Considerations 10

Lateral Flow Assay Design 11

- Step #1: Select Nanoparticle 13
- Step #2 Antibody Screening 17
- Step #3: Conjugate Preparation 20
- Step #4: Membrane Selection 21
- Step #5: Membrane Striping 22
- Step #6: Sample Pad Selection 24
- Step #7: Conjugate Pad Selection 26
- Step #8: Wick Selection 27
- Step #9: Drying Conjugate onto Conjugate Pad 27
- Step #10: Card Assembly 28
- Step #11: Cutting Into Strips 28
- Step #12: Assay Execution 29
- Step #13: Analyzing the Strip 30
- Step #14: Assay Optimization 31
- Step #15: Cassette Optimization 33
- BasicTroubleshooting 33
- Frequently Asked Questions 34
- Conclusions: 37

Introduction to Lateral Flow

OVERVIEW

Lateral flow assays (LFAs) are rapid and inexpensive diagnostic tests with long shelf lives that don't require refrigerated storage. This simple assay format provides results without additional processing of the sample or external equipment, and with minimal training. Their simplicity is a significant advantage and 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 label. The faint red line indicates that the subject is pregnant. The darker red line indicates that the test was valid.

With their low cost and simplicity, billions of tests are produced each year to test for a variety of different analytes. In the most common configuration, a colored line at the test location indicates a positive test (**Figure 1**). A second line at the control location indicates that the test was valid.

The red color of the lines is from colloidal gold nanoparticles bound to the test and control lines. These colloidal 40 nm gold nanoparticles have become the industry standard for lateral flow assays. The red color stems from an optical plasmon resonance of the 40 nm gold nanoparticles, which strongly absorb green and blue light. nanoComposix has developed a wide variety of high quality plasmonic nanoparticles with various optical signatures, including both 40 nm gold and other unique nanoparticles which give rise to increased sensitivity in lateral flow assays (**Figure 2**). These new probes will enable lateral flow tests with increased sensitivity and reproducibility. Such ultra-sensitive diagnostics can be quantified with inexpensive digital readers (e.g. cell phones). We believe this combination has the potential to revolutionize the point of care diagnostic industry.

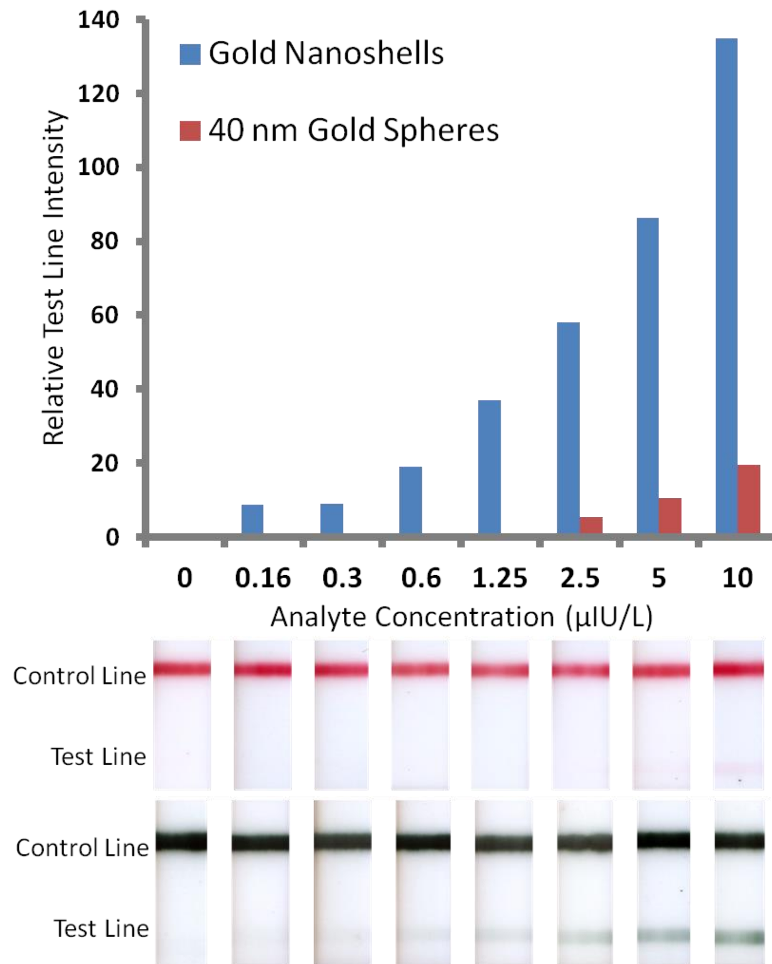

Figure 2: Top Lateral flow assay with serial dilutions of hormone analyte showing the increase in sensitivity when using gold nanoshells (blue) as the probe over 40 nm spherical gold (red).

LATERAL FLOW COMPONENTS

The lateral flow test is assembled from a few key components:

- a sample pad (for sample application)
- a conjugate pad (containing dried colored nanoparticles)
- a nitrocellulose membrane (striped with a test and control line)
- a wick pad

Often the strip components are housed inside of a plastic cassette so that only sample pad and test and control lines are visible. The LFA components are held together on a plastic adhesive backing card with a carefully controlled overlap, allowing for unimpeded capillary flow of the sample from the sample pad through the conjugate pad, nitrocellulose and into the wick. 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 (2-15 minutes) indicates the presence or absence of the target analyte (**Figure 1**).

At the core of a lateral flow assay are the "conjugates", also called detector nanoparticles or probes. These are one of nanoComposix's core competencies. These are brightly colored nanoparticles are connected to antibodies that recognize the target test compound 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 capture line, which contains an immobilized protein that either binds to the target analyte or competes with the target analyte for binding, and a control line that contains an antibody that binds to the antibody on the surface of the particle, regardless of the presence or absence of analyte, to confirm that the assay is working correctly (**Figure 3**). 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 neutralize the sample. The liquid wicks to the conjugate pad which contains the dried nanoparticle conjugates. The conjugate is rapidly 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 the analyte was detected or not.

ASSAY FORMATS

Lateral flow assays (LFA) can detect a wide range of targets. 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. The two common formats are "sandwich" and "competitive" which are described below:

SANDWICH FORMAT

The sandwich assay format is typically employed for detecting relatively large analytes. If the analyte has at least two distinct binding sites, a "sandwich" assay can be developed where an antibody to one binding site on the analyte is conjugated to the nanoparticle and an antibody to another binding site is used for the test line. If the analyte is present in the sample, the analyte will become the "meat" of the sandwich binding the

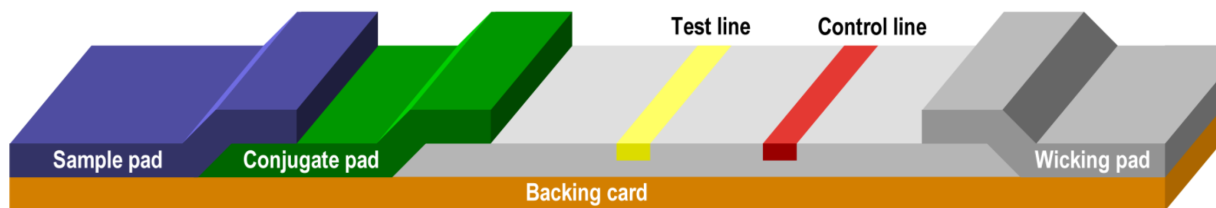

Figure 3: Schematic of a generic lateral flow test. The **sample pad** absorbs the sample and transports the sample to the conjugate pad. The **conjugate pad** contains the dried down antibody-nanoparticle conjugate. The nitrocellulose membrane has test and control lines that show the assay results.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

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 where 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 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, yielding 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.

Both assay formats utilize 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 can be trusted. 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 clearly visible regardless of the presence or absence of analyte in the sample. The lack of a control line indicates problems with the test itself and therefore invalidates the results.

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 strength of the test line can provide "semi-quantitative" results, where the result is reported as falling within bins of 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 in most cases, a digital reader. Recent advancements in the development of inexpensive readers based on cell phone technologies have recently become available (**Figure 4**) and provide a simple and inexpensive solution for quantifying lateral flow assay results.

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.

The methods and techniques used to conjugate antibodies to the surface of nanoparticles are critical for optimizing the performance of lateral flow assay tests. For gold nanoparticles, antibodies can either be physisorbed to the surface, referred to as passive adsorption, or they can be covalently coupled. 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. We typically purify all antibodies 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.).

Figure 4: (A) i-calQ smartphone reader with strip holder attachment. (B) Novarum hardware-free reader.

PASSIVE ADSORPTION

This is the original 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 antibody loading sweep can be performed to identify conditions where antibody absorption 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 post conjugation. There are two main drawbacks to the passive adsorption. Firstly, every antibody requires slightly

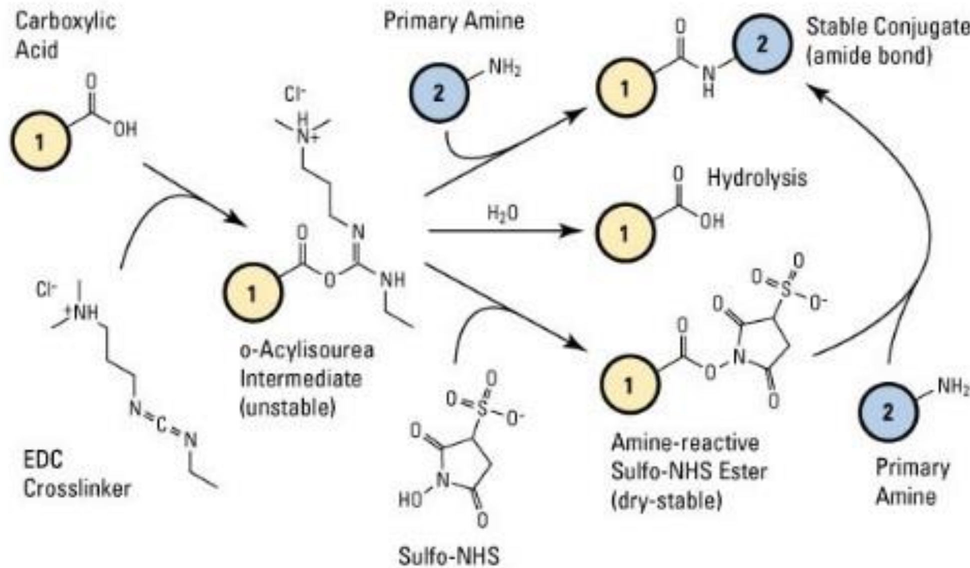

Figure 5: EDC-NHS reaction scheme – from [Thermo-Fisher].

different conditions. Secondly, some antibodies may detach from the nanoparticle surface which can lead to a decrease in sensitivity and variable results.

COVALENT COUPLING

Increasingly, LFA developers are covalently binding antibodies to the surface of nanoparticle lateral flow probes. Covalent attachment is more stable with less antibody desorption and requires less antibody during conjugation. Covalent attachment can be accomplished with several different chemistries. For our BioReady products that are optimized for lateral flow, we typically utilize amide bonds to connect a carboxylic acid functionalized nanoparticle to free amines on the antibody. This covalent bond is achieved through an EDC/Sulfo-NHS intermediary (**Figure 5**) generated from a carboxylic acid surfaced particle. 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 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 decisions to be made. 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.

A critical first step is to identify a control assay, whether in lateral flow or on a different platform, that can independently measure the analyte of interest and to validate the assay if your assay is intended for clearance through regulatory agencies such as the FDA. Next, the antibody for conjugation needs 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 guidebook. 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 what to consider 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 card based LFA manufacturing process at nanoComposix is shown in Figure 6. Small scale production runs use backing cards that are 30 cm long and can be cut into ~50 strips with a 5 mm width. The first step in the process is to prepare conjugates (nanoparticle + antibody) and transfer the conjugates to an appropriate solution for spraying and drying onto the conjugate pad (**Figure 6, Step 1**). For highly reproducible striping of test and control lines, a dispenser with a flexible hollow glass fiber connected to a syringe pump is used (**Figure 6, Step 2**). The conjugate is applied to the glass fiber conjugate pad using a non-contact spray head on the dispenser (**Figure 6, Step 3**). Once the solutions are applied to both the nitrocellulose membrane and the conjugate pad, these components are dried and cured in the 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 attached to self-adhesive backing cards using a clam shell laminator. To assemble the 30 cm master cards, the nitrocellulose is applied first, followed by the conjugate pad, wick pad and then sample pad (**Figure 6, Step 4**). The laminator ensures an accurate placement of master card components with the correct overlaps and controlled pressure. The assembled cards are then cut into individual strips with a guillotine (**Figure 6, Step 5**) and assembled into a plastic cassette which is sealed in a foil lined bag with desiccant (**Figure 6, Step 6**). Each of these steps is described in more detail in the sections below.

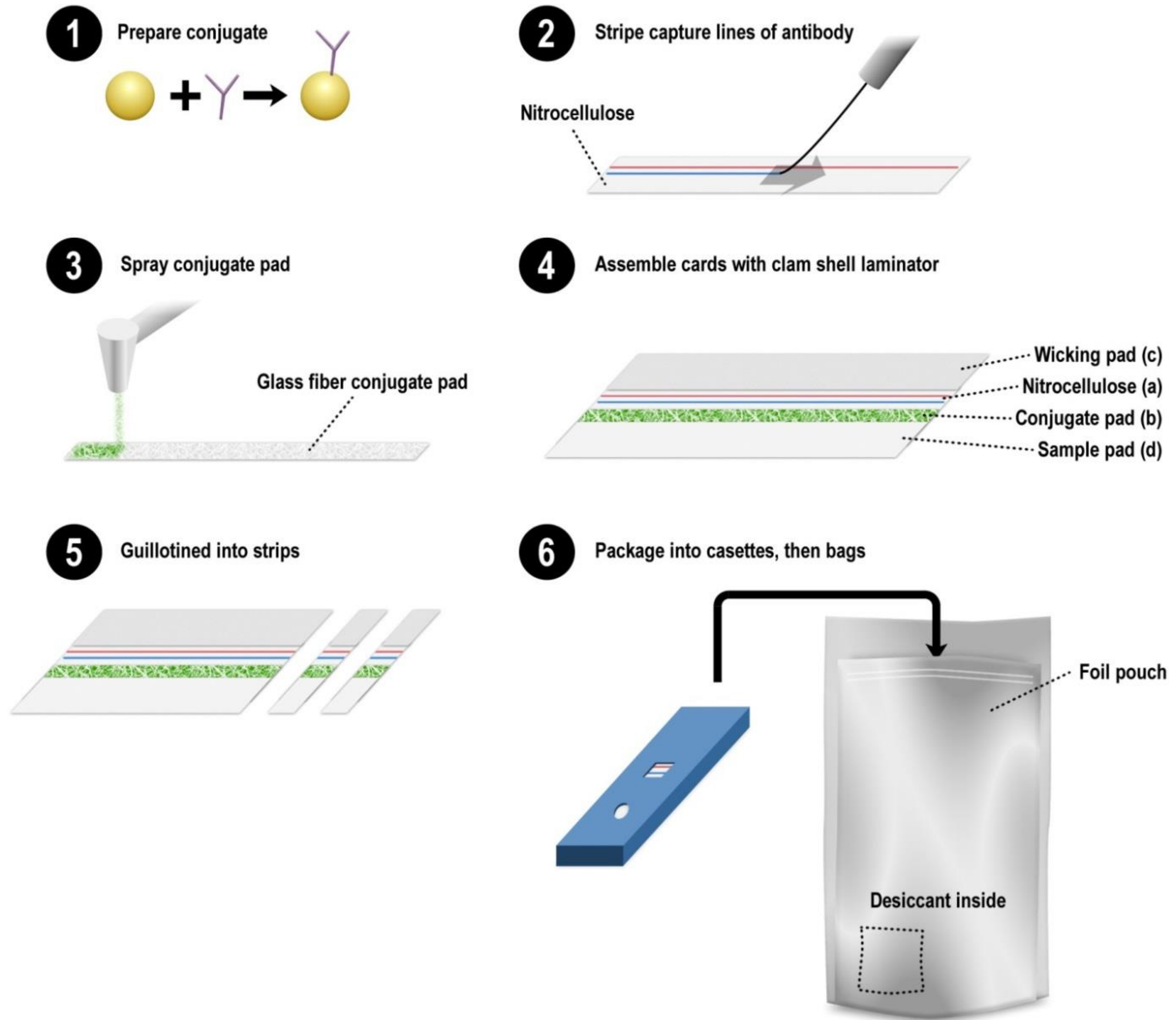

Figure 6: General procedure for the card-based assembly of a lateral flow device consisting of conjugate preparation (1), striping of capture lines (2), spraying conjugate pad (3), assembly of cards (4), strip cutting (5) and the packaging into cassettes (6).

Step #1: Select Nanoparticle

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

BIOREADY 40 NM CARBONATE GOLD

Our BioReady 40 nm carbonate gold nanoparticles can be conjugated to proteins through passive adsorption (also referred to as physisorption). 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 40nm carbonate gold is provided at an optical density (OD) of 4 at pH ~8.0-8.5 which is suitable for many IgG antibodies. A pH titration can be performed to optimize the pH of conjugation.

PROS:

- 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

CONS:

- 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 CARBOXYLIC GOLD

nanoComposix BioReady 40 nm carboxylic (-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.

PROS:

- Whole antibodies, antibody fragments and small molecules can be irreversibly bound
- Generally less antibody is required than for passive adsorption
- Stable, irreversible amide bond formed
- Improved control over antibody/particle loading (difficult to accomplish in passive adsorption because of colloidal stability issues)

CONS:

- Requires an additional step to activate the -COOH surface with EDC/Sulfo-NHS chemistry

BIOREADY 40 NM NHS GOLD

nanoComposix BioReady 40nm 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 40nm 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.

Pros:

- 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

Cons:

- NHS gold solution must be used immediately upon resuspension
- At larger scales, it is more cost effective to perform EDC/NHS chemistry with nanoparticles having carboxylic acid surfaces and in some cases, performing EDC/NHS chemistry immediately before antibody binding can increase assay sensitivity.

BIOREADY 150NM CARBOXYLIC 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 (**Figure 7**). While 40 nm gold has historically been the nanoparticle of choice for lateral flow assays, we have found that gold nanoshells, another type of plasmonic nanoparticle, can dramatically increase the sensitivity of lateral flow assays. The gold nanoshells of choice 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 each particle has a higher optical density relative to 40 nm gold particles, fewer binding events are required in order to see the test line in a lateral flow test **(Figure 8)**. 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.

PROS:

- Up to 20X increase in lateral flow sensitivity
- NHS and COOH based covalent linkage chemistry available

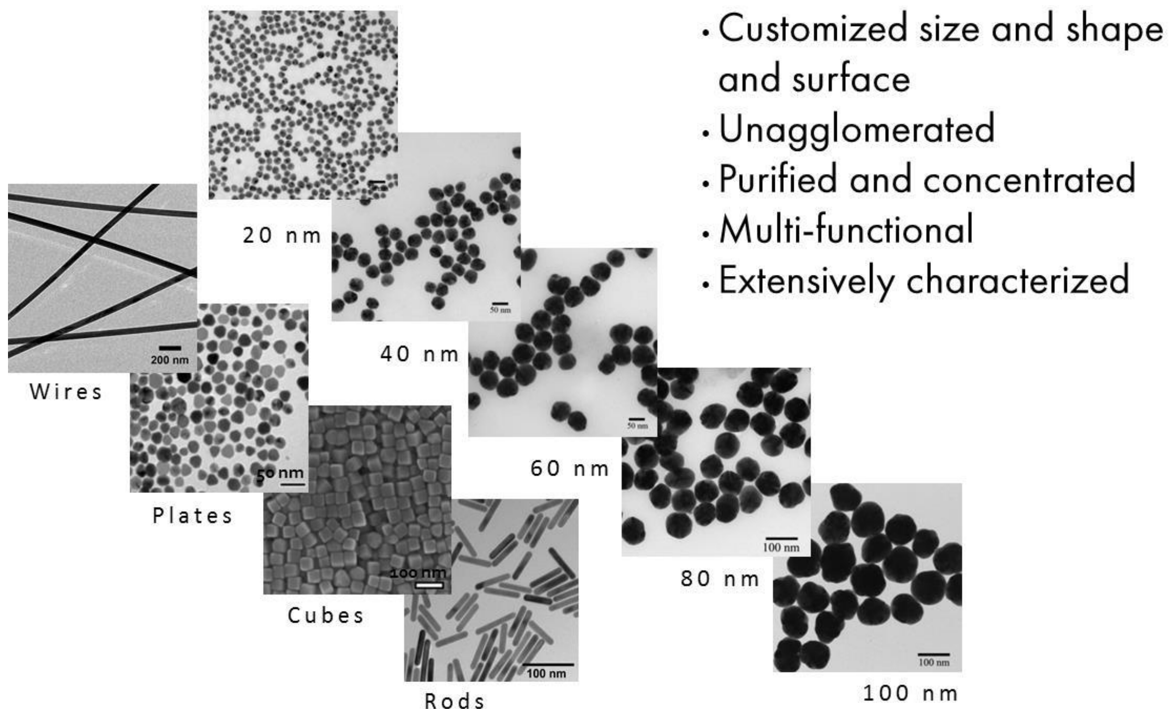

Figure 7: Metal nanoparticles with various sizes and shapes produced at nanoComposix.

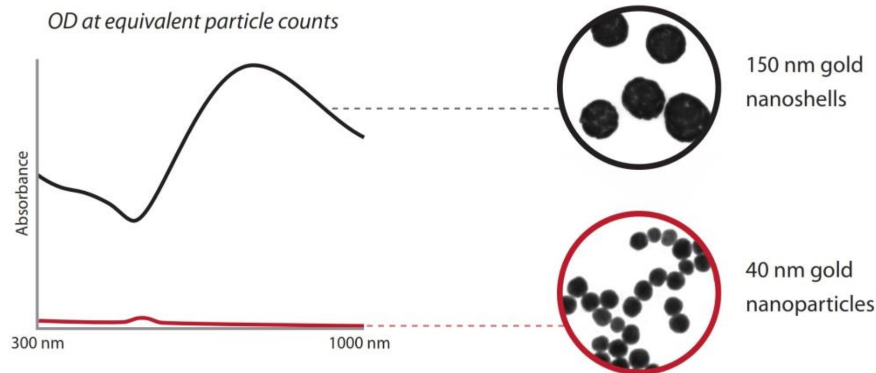

Figure 8: Optical extinction measurements for a 40 nm gold nanoparticle vs. a 150 nm diameter gold nanoshell.

CONS:

- 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 not based on gold utilized in lateral flow assays. 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 also have stability issues in certain matrices. 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, 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 Screening

Selection of the optimal antibodies is a critical aspect of lateral flow assay design. The ultimate 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 and provide a rapid visual readout to the end user. It is important during initial development to ensure that you select the optimal antibody pair. 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, the reagents screened will consist of one antibody and an analyte-protein conjugate.

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 whether the antibody is monoclonal or polyclonal are all important factors when selecting an antibody. The first step is to research what antibodies are available for your specific application. The number of antibodies available for a specific analyte varies greatly. If an antibody is not available for the assay that you would like to develop, custom manufacturing of an antibody should be initiated as soon as possible. Typically this involves the injection of an antigen into a carrier animal followed by affinity screening of the antibody in drawn blood. If antibodies are commercially available, the antibody selection may only include a few options, or there may be large selection of antibodies to choose from. As many antibodies as possible should be acquired to perform initial screening to determine which are most effective.

Typically a mixture of monoclonal and polyclonal antibodies will be selected for the first round of screening. Monoclonal antibodies are given a clone number, which indicates that the antibodies are from a single clone of hybridoma cells. Monoclonal antibodies with the same clone number can be available from multiple distributors. It is important to look at the clone number when selecting antibodies to avoid screening the same antibody twice. In some circumstances, distributors will change the clone number or not list the clone number on the certificate of analysis. If possible, determine if the clone number from the distributor is the same as a different clone number from the actual manufacturer to save on cost and time of screening. Other considerations for down-selecting antibodies are the cross-reactivity characteristics, 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 to ELISA 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.

Antibody selection is assay specific and it is recommended to spend enough time and effort to confidently determine the best pair since the cost and challenges of switching antibodies at later stages in the development is significant. 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 to test (**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.

	Ab #1 on particle	Ab #2 on particle	Ab #3 on particle	Ab #4 on particle
Ab #1 on strip		X	X	X
Ab #2 on strip	X		X	X
Ab #3 on strip	X	X		X
Ab #4 on strip	X	X	X	

Table 1: Antibody (Ab) evaluation matrix.

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

	POLYCLONAL	MONOCLONAL
ADVANTAGES	• Inexpensive to produce • High affinity • Will recognize multiple epitopes (generally provides more robust detection) • Polyclonal antibodies are often the preferred choice for detection of denatured proteins • Higher tolerance for differences in antigen (i.e. glycosylation of proteins)	• Constant and renewable source, all batches will be identical • Less background relative to polyclonal antibodies • Homogeneity is very high, ensuring 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)

Table 2: Comparison of advantages and disadvantages of using polyclonal and monoclonal antibodies.

the high affinity and ability to recognize multiple epitopes. However, the lateral flow assay is not limited to monoclonal/polyclonal formats. 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 will most likely not be 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. Since 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 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 280nm, 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 should be a secondary antibody specific to the species of the conjugated antibody. For example, a mouse monoclonal antibody is often used as the capture antibody on 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: Conjugate Preparation

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. Typical starting ranges for antibody concentration are 5 µg/OD.mL for passive adsorption to 40 nm carbonate gold, 2.5 µg/OD.mL for covalent attachment to 40 nm carboxylic gold, and 1µg/OD.mL for covalent attachment to 150 nm carboxylic gold nanoshells. In some cases, the optimal antibody

concentration for covalent binding is lower, further reducing the cost of assay reagents. 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 at:

BIOREADY 40 NM CARBONATE GOLD: PASSIVE ADSORPTION
BIOREADY 40 NM CARBOXYLIC GOLD: COVALENT CONJUGATION
BIOREADY 40 NM NHS GOLD: SIMPLIFIED COVALENT CONJUGATION
BIOREADY 150 NM CARBOXYLIC 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-conjugations to our 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.

Step #4: Membrane Selection

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), reduces 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.

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

Table 3: Table showing relationship between membrane selection, flow time, pore size, and sensitivity.

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 manufactures.

Step #5: Membrane Striping

The next step in the lateral flow assay design process is to stripe the test and control antibody lines onto the nitrocellulose membranes (**Figure 9**). At nanoComposix, we use an Imagene Isoflow dispenser, 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 the test and control lines but rather a controlled humidity environment of ~50% RH since nitrocellulose that is too dry results in spotty, non-uniform lines while nitrocellulose that is too damp will result in a widened test line which decreases the signal intensity. Always refer to the storage and handling guidelines provided by the manufacturer. Once the nitrocellulose has been

striped and dried, it is important that the humidity be kept constant and low (less than 20%) until the test strips are sealed in pouches with desiccant.

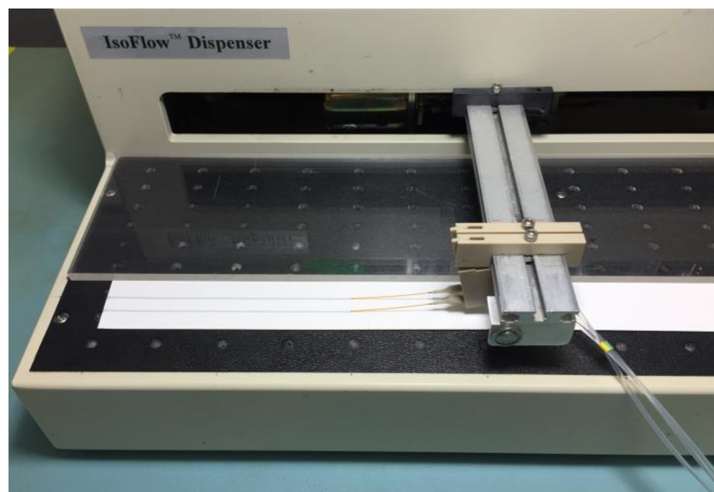

Figure 9: Test and control deposition with Imagene Isoflow dispenser.

Important parameters for striping include the reagent concentrations and the dispense rate, which will depend on the specific assay reagents and the membrane speed being used. Typical dispense rates are between 0.5 and 1 µL/cm providing 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 required. For competitive assays, an analyte-protein conjugate (i.e. drug analyte-BSA) is dispensed at the test line rather than an antibody. Analyte-protein conjugates tend to spread 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.

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 1.5 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). The antibodies used for striping do not need to be purified from preservatives, and can be diluted in 1X PBS buffer. Adding 0.5-1% trehalose may be added to striping solution to increase the stability of the protein binding on the membrane.

After striping the membranes, it is important to maintain a system that keeps track of which line is the test line and control line before they are dried and no longer visible. We recommend using a pen or pencil to mark each membrane at the end with the line location and a "T" or "C." Although this may seem trivial, it will ensure that the membrane will be placed in the right orientation when assembling your test strips. It is also important to mark any parts of the membrane where striping may have been inconsistent while wet and visible (possibly from an air bubble in the line) so that these strips will be discarded (refer to Step #10 below for strip assembly). Membranes should be dried in a forced-air convection oven for 1 hour at 37 °C, followed by overnight storage in a desiccated environment. We store our striped membranes overnight with a relative humidity of <20% to fully cure before testing or final packaging.

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 a time consuming step during the optimization process as well as the long term manufacturing at the large scale. 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. We do not recommend manual striping. If you do not have access to a striper, NanoComposix can stripe your membranes as a custom service. Contact us at info@nanocomposix.com for more details.

Step #6: Sample Pad Selection

The sample pad is the first material that comes in contact with the sample when running a lateral flow assay. Sample pad materials and pre-treatments should be screened to ensure that the sample has the optimal flow and treatment before the sample reaches the antibodies in the system.

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 "neutralizing" the samples before reaching the conjugate pad 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 employed 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 10).**

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 required 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 screen 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.

As previously mentioned, treating the sample pad with an optimized buffer can enhance assay performance. The treatment can mitigate sample variability (pH, viscosity, protein concentration, salt concentration, etc.), and improve 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 increased salt and surfactant concentrations, the mucins and proteins can be broken down decreasing viscosity and

Figure 10: Lateral flow assay with sample pad that has a red blood cell filter. Image from mdimembrane.com/

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.

Sample pad treatment can be performed by immersion, or by spraying uniformly with an automated dispenser (Isoflow or Biodot). Spraying will give 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 #7: Conjugate Pad Selection

It is important to choose a conjugate pad 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. Conjugate pads made of glass fibers are generally recommended.

There are many materials available for use as a conjugate pad from multiple suppliers, such as Millipore and Ahlstrom. The material chosen 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. Millipore GFDX is more dense material and works well for most sandwich assays. This material can hold a relatively larger volume of conjugate and has a slower release. Both of these properties 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 faster 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.

Step #8: Wick Selection

The wick is an extremely important component of the lateral flow test that is often not appropriately optimized. 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 must prevent backflow of the excess reagents for as long as possible (at least past the read time of the test). Select a material and size that has an absorption capacity much higher than the sample and running buffer volume. Materials such as Whatman 470 and Ahlstrom 222 are recommended starting materials. 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 #9: Drying Conjugate onto Conjugate Pad

Typically, conjugate is 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 (i.e. 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 any 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 down conjugate and re-solubilization once it interacts with the sample. A recommended starting concentration of sugars for 40 nm gold 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 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 should be dried in a forced-air convection oven for 1 hour at 37˚C. The dried conjugate pads should be cured overnight in a desiccated environment with <20% humidity prior to testing.

Step #10: Card Assembly

NanoComposix's employs 30 cm backing cards (e.g. Lohmann) for assembling the lateral flow tests (**Figure 11**). These cards have a self-adhesive coating underneath a series of peel off covers. To attach the various components of the test strips the covers are removed one by one and the various components stuck to the backing card (see **Figure 6 (4)**). Until test volumes are at millions of tests per year, building the assays on cards rather than reel to reel is the simplest and most economical route. Once you have all of your components, you are ready to assemble your lateral flow device. This can be done by hand or with a laminator. The laminator registration pins specify exactly where each component will be placed and ensures reproducible assembly onto the adhesive backing card. Clam shell laminators are relatively inexpensive and greatly reduce variability of component placement, in turn generating more reproducible test results with lower coefficients of variation.

Figure 11: 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

Step #11: Cutting Into Strips

Once a master card is assembled, the next step is to cut the card into strips. Strip widths may vary between 3 mm and 6 mm. 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. While strips can be cut by hand, an automated guillotine should be used to accurately cut the strips with a high degree of reproducibility.

Step #12: Assay Execution

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.

HALF STRIP ASSAYS (LIQUID CONJUGATE)

For initial screening of antibodies, we recommend starting with half strip assays where the strip is prepared without a sample pad 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 12**). 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, half strip 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.

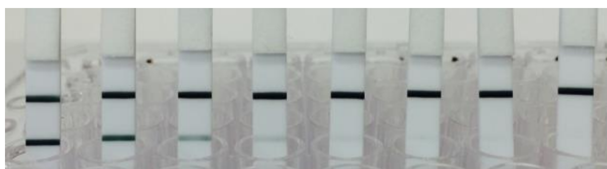

Figure 12: Running liquid conjugate/half strip assays.

FULL ASSAYS (DRIED CONJUGATE)

Once the conjugate is dried down and the strips are fully assembled (through Step #11), 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 utilized 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. By testing with a clean system, the initial limit of detection can be determined

and provide a metric for comparing different antibody pairs, nanoparticle selection, test strip materials and buffers.

Once 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 differ. 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 does not contain the analyte endogenously. 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.

Step #13: Analyzing the Strip

When analyzing the test strip, there are many methods that can be utilized, and choosing the appropriate method will depend on the stage of development and whether the assay is intended to be a qualitative or quantitative assay. For effective optimization it is important that you have an objective means of quantifying the output of the test strips to determine if you are doing better or worse.

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've also produced 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.

Alternatively, 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 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 #14: 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 and conjugate pad material and treatment, nitrocellulose membrane, test line concentration, wick pad material, running buffer, cassette, and sample volume. 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 13).** When optimizing an assay, the 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. This section describes some of the optimizations that are routinely performed at nanoComposix.

CONJUGATE PREPARATION: ANTIBODY INCUBATION TIME SCREENING

For covalent conjugations we generally recommend starting with a 2 hour incubation time. During optimization, shorter and longer incubations should be tested. In circumstances where you are limiting the number of antibodies per particle rather than saturating the surface (i.e. 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, in turn decreasing antibody functionality.

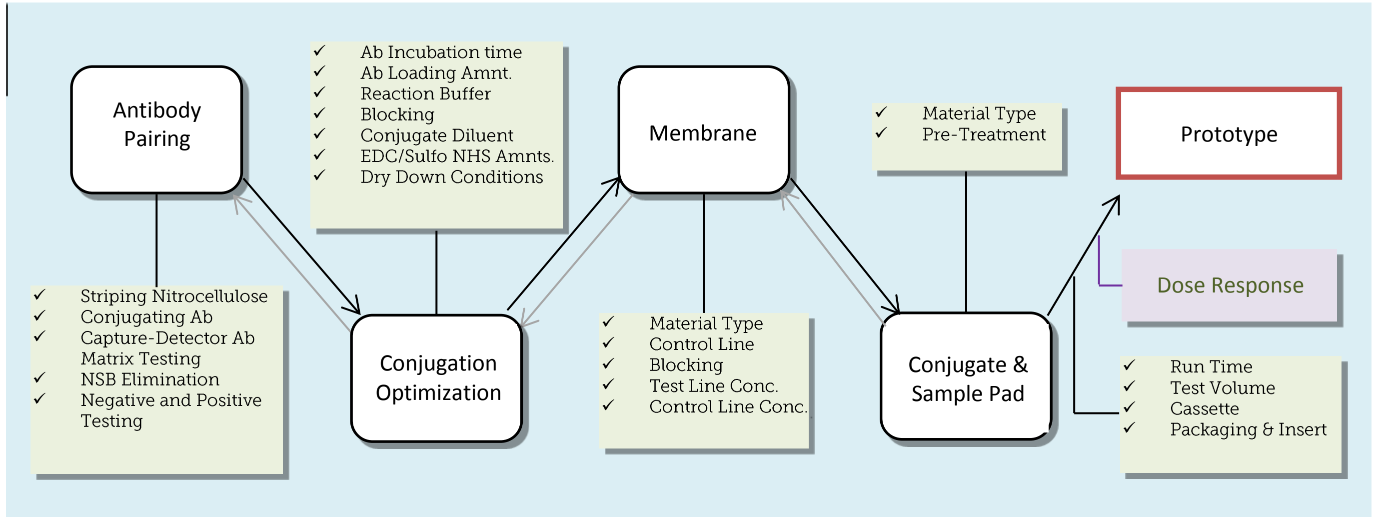

Figure 13: Iterative optimizations that are performed to maximize performance of lateral flow assay.

CONJUGATE PREPARATION: ANTIBODY TO GOLD RATIO

Covalent conjugation is a great choice if you'd like to control the amount of antibody per particle. Our carboxylic gold nanoshells can be loaded with 1-25 µg IgG/20 OD.mL. Our 40 nm carboxylic gold can be loaded with 2-50 µg IgG/20 OD.mL. When decreasing the number of antibodies on the surface, it may be desirable to use a short 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 etc.) any remaining NHS-esters prior to processing to avoid crosslinking of particles.

CONJUGATE DILUENT COMPONENTS

Conjugate diluent components will vary significantly depending on sample media. These will need to be investigated and optimized when moving from a clean system (analyte spiked into buffer) to a clinically relevant sample. We recommend using real samples as early as possible during development. For a test using gold nanoshell conjugate at 20 OD and a 15-20 µL volume of plasma or serum as a sample, a suitable starting point for the conjugate diluent is 0.5x PBS, 0.5% Casein, 0.5% BSA, 1% Tween-20, 10% Sucrose, and 5% Trehalose. Tween is typically reduced if non-specific binding is observed, and increased if there is reduced signal intensity at the test and control lines. Other components will likely need to be optimized for each individual assay. If high concentrations of detergents are required, it is recommended to use the more robust, covalent attachment for your conjugate so that the detergent doesn't negatively interfere with colloidal stability.

It is important to introduce your analyte in its matrix early in assay development. Obstacles generally arise when switching from a "clean" analyte + buffer system to using actual clinical samples (saliva/plasma/stool etc.). Most sample matrices are very complex and will require investigating blocking agents and detergents in an effort to minimize sample variation.

Step #15: Cassette Optimization

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 taking another path. 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. For initial testing, existing generic cassettes may be sufficient.

Basic Troubleshooting

UNUSUAL RUNNING FRONT

A "V" shaped running front (instead of a uniform line running across the membrane) could be caused by too high of a salt concentration when striping antibodies, an issue with the pre-treatment of conjugate pads, or poor flow control/streaking of conjugate during deposition.

DECREASE/LOSS OF TEST AND CONTROL LINE WHEN SWITCHING FROM "CLEAN" (ANALYTE SPIKED IN BUFFER) SYSTEM TO CLINICAL SYSTEM

A decrease of 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 or conjugate pad pre-treatment buffer can help recover the signal intensity.

FALSE POSITIVE

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. In order 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).

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 carboxylic 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 PURCHASING CARBOXYLIC NANOPARTICLES OVER NHS NANOPARTICLES?

The NHS nanoparticles are a great tool for sweeping 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 most notably 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 PURCHASING CARBOXYLIC ACID 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 AND OTHER APPLICATIONS?

Our 150 nm gold nanoshells are 30X visibly brighter per particle than traditional 40 nm gold used in lateral flow. Because they have been engineered with a glass 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 IS THE OPTIMAL CONJUGATION TIME?

Generally, we recommend starting with a 2 hour incubation time. During optimization, shorter and longer incubations should be tested. In circumstances where you are limiting the number of antibodies/particle rather than saturating the surface (i.e. competitive assays) we generally recommend a shorter incubation time (as short as 5 minutes) to reduce the chances of antibodies folding and binding to several available acid groups on the surface and in turn decreasing antibody functionality.

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.

> For example: Weigh out between 1-10 mg EDC into a tube and bring final concentration to 10 mg/mL in water just prior to conjugation. If the mass of EDC is 8.6 mg, add 860 µL water for a final concentration of 10 mg/mL.

For technical assistance, please contact (858) 565-4227 x2 or email us at info@nanocomposix.com.

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 and achieve low non-specific background 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 ready to help in any way that we can – just reach out to us at info@nanocomposix.com.