nanoComposix

High Sensitivity Conjugation Kit for Lateral Flow

Product Numbers:

GSZR150-10M

AUZR40-10M

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Introduction

Welcome to the nanoComposix **High Sensitivity Covalent Conjugation Kit for Lateral Flow** and thank you for taking the time to read this product guide! This kit contains all reagents and protocols necessary to covalently bind antibodies to the surface of BioReady™ carboxyl nanoparticles, including 40 nm Gold Spheres, 80 nm Gold Spheres, and 150 nm Gold Nanoshells. This kit is designed to help you rapidly fabricate and optimize covalent conjugates. Once you're familiar with the conjugation process you can purchase reagents separately and apply these procedures to any of nanoComposix's BioReady™ carboxyl nanoparticles. We strongly encourage you to carefully review the information and protocol included. We hope you will find the instructions here clear, simple, and easy to follow. If you have any questions, please email us at *info@nanocomposix.com* or call us at +1 (858) 565-4227. You may also contact your local distributor, if applicable.

WHY COVALENT?

Covalently binding antibodies to nanoparticle surfaces has several advantages compared to passive conjugation:

- Less antibody is needed to maximize sensitivity, reducing the overall cost of an assay.
- Covalent conjugates offer higher stability, which solves challenges associated with difficult sample matrices and harsh buffering conditions (high salts or detergents).
- The greater stability and reproducibility of covalently-conjugated antibodies reliably quantitates analytes that can be difficult or sometimes impossible to achieve with passive conjugation.
- Conjugates are easily and consistently prepared without requiring extensive salt concentration and pH titration

screens, which saves time when performing antibody screening experiments.

- The antibody-to-particle ratio can be precisely controlled, which is important for adjusting the dynamic range in competitive assays and optimizing sensitivity when using antibodies with different binding kinetics.

NanoComposix's BioReady™ products are optimized for use in lateral flow diagnostic assays. Carboxyl-functionalized nanoparticles are recommended for covalent binding. For this surface, EDC and sulfo-NHS reactive groups are used to form amide bonds that link carboxylic acids on the nanoparticle surface to primary amines in the lysine residues of the antibody or protein. For example, a typical IgG antibody will have 80–100 lysine residues, of which 30–40 will be accessible for EDC/NHS binding.

It's important to understand that each antibody is unique and may exhibit different performance than other proteins when conjugated. There is no one-size-fits-all conjugation method. NanoComposix has developed this kit to help find conditions that will optimize conjugate efficacy, stability, and reproducibility.

General Information

This kit contains all the components necessary to run at least **4 experiments** that will help you produce high quality conjugates. There is 1 antibody buffer exchange/desalting experiment, 3 conjugation experiments, and 1 optional blocking experiment. Before each experiment, read the instructions completely and carefully. If you have any questions, please contact us at *info@nanocomposix.com*.

STORAGE & STABILITY

Store kit at 2–8°C immediately upon receipt. Refer to the list of materials supplied for individual component storage.

MATERIALS SUPPLIED

Item	Amount	Storage
Buffer exchange/desalting column *10 kDa MWCO, for antibody buffer exchange/desalt*	1 each	2–25°C
Housing tubes for desalting column *For antibody buffer exchange/desalt*	2 each	2–25°C
Desalting buffer for antibody *10 mM potassium phosphate, pH 7.4*	10 mL	2–25°C
BioReady™ carboxyl gold nanoparticles, 20 OD	10 mL	2–8°C
Potassium phosphate reaction buffer *5 mM potassium phosphate, 0.5% PEG 20 kDa, pH 7.4*	50 mL	2–25°C

Item	Amount	Storage
Sodium phosphate reaction buffer *5 mM sodium phosphate, 0.5% PEG 20 kDa, pH 7.4*	50 mL	2–25°C
PBS reaction buffer, 50X stock *0.5X PBS, 250 mg/mL PEG20K*	1 mL	2–25°C
60 mL PETG reagent bottle, empty *For dilution of 50X PBS reaction buffer to 1X*	1 each	2–25°C
EDC	3 × 10 mg	2–8°C
Sulfo-NHS	3 × 10 mg	2–8°C
Hydroxylamine quencher	100 µL	2–25°C
NCX block buffer *4 mM sodium tetraborate, 1% BSA, 0.05% sodium azide, pH 8.2*	5 mL	2–8°C
NCX conjugate diluent *0.5X PBS, 0.5% BSA, 0.5% casein, 1% Tween 20, 0.05 % azide pH 8*	10 mL	2–8°C
1.5 mL microcentrifuge tubes *For conjugation*	20 each	2–25°C
NCX lateral flow running buffer *1X PBS, 1% Tween 20*	10 mL	2–25°C

MATERIALS REQUIRED & NOT SUPPLIED

- Microcentrifuge
- Single channel pipettes capable of pipetting 5–1000 µL
- Orbital or end-over-end rotator or mixer for incubation steps
- Freshly dispensed deionized water*

  * DI water that has been stored for long periods of time can have a low pH. We recommend freshly-dispensed DI water for use in conjugation and dilution buffers.

- Method for determining protein concentration (A_{280}, BCA, Bradford Assay)

ADDITIONAL RECOMMENDED MATERIALS

- UV-Vis spectrophotometer
- Sonicator
- Calibrated pH probe
- Lateral flow test strips for functional QC testing of conjugate

Antibody Buffer Exchange Protocol

BACKGROUND

Covalent conjugation using EDC and sulfo-NHS requires the antibody to be suspended in an **amine-free buffer** that will not interfere with conjugation to the primary amines of the antibody. During development and processing many antibodies are purified via protein A, protein G, or other affinity purification methods. Tris buffer is commonly used to elute antibodies from purification columns, and sodium azide is often added as a preservative. Because they contain amine groups, **both azide and Tris need to be removed** prior to covalent conjugation.

The antibody buffer exchange step (also knowns as desalting) is the process of washing out the interfering salts (e.g. azide, Tris) and exchanging the antibody into a different buffer suitable for covalent conjugation. Unless you are confident that the antibody is in an amine-free buffer, we recommend that all antibodies be desalted and exchanged into our 10 mM potassium phosphate buffer before conjugation.

This kit includes a purification column with a 10 kDa molecular weight cut-off to concentrate, wash, and resuspend your antibody to a concentration of ≥ 1 mg/mL in amine-free buffer.

Note: *BSA and other stabilizing proteins will also need to be removed. However, these interfering components may be ≥ 10 kDa and cannot be filtered out with the provided column. You may have to purchase a larger membrane filter cutoff separately (not to exceed the size of the conjugating antibodies) or purchase affinity purified antibodies free of any stabilizing proteins.*

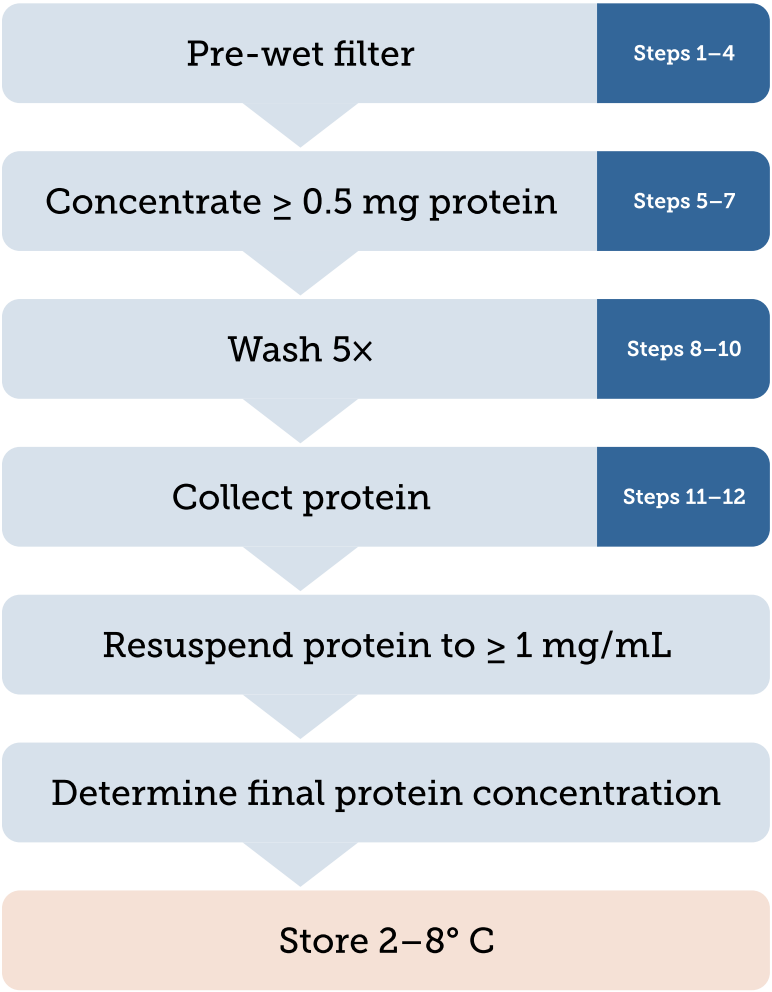
TECHNICAL NOTES

- This kit provides 10 mM potassium phosphate pH 7.4 buffer to wash and desalt your antibody. If the antibody is supplied in a buffer that has a significantly different pH than 7.4, an alternative desalting buffer may produce better results. We recommend contacting the antibody vendor to select an appropriate buffer for this step.
- It is recommended to desalt/exchange **at least 0.5 mg of material** for use in the subsequent conjugations to nanoComposix BioReady™ carboxyl gold particles.
- **Perform all steps as quickly as possible (< 90 minutes).** Leaving concentrated protein in the membrane filter for an extended period may result in low yields. If possible, perform this step in a refrigerated centrifuge at 4°C.
- A very small volume of solution containing the antibody should remain in the filter after each spin (~50–100 µL). **Spinning until all the solution has gone through the filter may cause adsorptive loss of antibody on the membrane.**

MATERIALS

- Buffer exchange/desalting column (Millipore Amicon® Ultra 0.5 mL, 10 kDa, Cat# UFC501096)
- 2 × 2 mL housing tubes for desalting column
- Antibody desalting buffer
    - 10 mM potassium phosphate, pH 7.4

ANTIBODY BUFFER EXCHANGE FLOW CHART



ANTIBODY DESALTING/BUFFER EXCHANGE PROTOCOL

1. *Recommended:* Confirm that the antibody concentration matches that provided on the antibody Certificate of Analysis using A_{280}, BCA, or a Bradford protein assay.

2. Pre-wet the filter device by adding 450 µL of the 10 mM potassium phosphate* (desalting buffer) to the filter and centrifuge for 5 minutes at 13.8k RCF.
   * Or choose another appropriate desalting buffer for your antibody (see *Technical Notes*, page 7).

   Note: *Do not touch the inside of the filter with the pipette as this may risk puncturing the filter.*

3. Carefully remove the filter device from microcentrifuge tube and dispose of filtrate located at the bottom of the microcentrifuge tube. Place the filter device back into the tube after the filtrate has been removed.

4. Add antibody solution into the filter and close cap.

   Note: *The filter can hold up to 500 µL. If the volume of antibody exceeds this capacity, you can centrifuge 450 µL of antibody solution in the filter first to concentrate and then add additional antibody before continuing with the wash steps. If the antibody volume is minimal before any centrifugation step, add desalting buffer to bring the total volume up to ~450 µL.*

5. Centrifuge 5 minutes at **13.8k RCF** to concentrate.

6. Remove the tube from the centrifuge and remove the filter device from the tube. Remove filtrate.

   Note: *It is recommended to retain the filtrate until after you have determined the yield. In the event of a ruptured filter, you may be able to recover your antibody from the filtrate.*

7. Place the filter containing the concentrated antibody back into the tube and add 350 µL of desalting buffer to the filter.

8. Centrifuge for 5 minutes at **13.8k RCF** to wash and concentrate. Remove filtrate after spin.

9. Repeat washing procedure (Steps 7–8) an additional four times for a total of **five** washes.

10. Following the final wash, turn the filter device upside down in a **new, clean** microcentrifuge tube (provided).

11. Centrifuge 5 minutes at **1.0k RCF** to collect purified and concentrated antibody.

    Note: *The microcentrifuge tube cap may be cut off with scissors before spinning.*

12. Bring the collected antibody solution to a final volume such that your antibody concentration is ≥ 1 mg/mL for storage (*recommended*).

    Note: *After purification, it may be desirable to aliquot and store purified antibodies ≥ 1 mg/mL at* ***recommended storage temperature*** *to minimize risk of contamination and to minimize freeze/thaw cycles. Antibody and protein stability conditions vary.* ***Always refer to the protein data sheet provided by the supplier for proper storage and handling.***

DETERMINE FINAL PROTEIN CONCENTRATION

Determine the antibody concentration before and after desalting to determine yield. It is recommended to use the same metric of quantification that was provided with the protein data sheet from the supplier.

Your protein is now ready for use in covalent conjugation and subsequent conjugate optimization. Again, store antibody at 2–8°C for short-term storage or –20°C to –80°C (aliquot and freeze for long-term storage).

Conjugation Protocol

BACKGROUND

This protocol is designed to be a simple and effective starting point to produce robust covalent conjugates using BioReady™ nanoparticles. The materials and steps provided will help avoid typical problems encountered when performing covalent conjugation (see *Common Pitfalls to Avoid* on page 33).

Because each antibody and assay are unique, certain parameters may need further adjustments to meet assay specifications. *Common Issues & Solutions* on page 18 provides additional guidance to help you overcome challenges with your assay..

TECHNICAL NOTES

- 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. Rehydration and addition of EDC & sulfo-NHS should be completed as quickly as possible (no longer than 5 minutes) to minimize hydrolysis of the NHS ester in water and ensure conjugation efficiency. PEG 20 kDa is included in the buffer formulation to help re-disperse the pelleted particles. It is not required but is

recommended, especially if you do not have access to a bath sonicator.

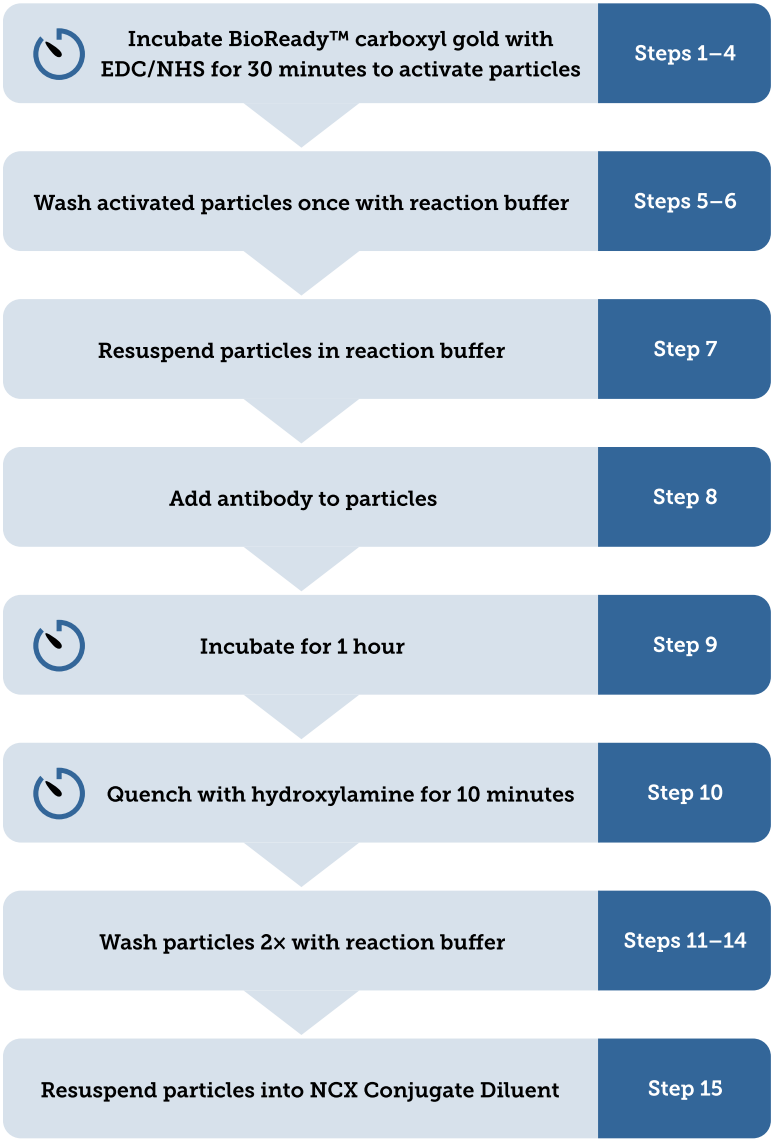
BEFORE STARTING THE CONJUGATION

- Make sure the antibodies are in a buffer free of amines (e.g. Tris) or any preservatives (e.g. sodium azide). See previous section on *Antibody Buffer Exchange*, page 6.
- Perform the conjugation in the 1.5 mL centrifugation tubes provided with the kit. Other types of tubes may contain plasticizers that could negatively impact the conjugation. See *Common Pitfalls to Avoid* on page 33 for more information.

MATERIALS

- 1.5 mL LabCon® microcentrifuge tube
- 1 mL BioReady™ carboxyl gold particles, 20 OD
- Reaction buffer: 5 mM potassium phosphate, 0.5% PEG, pH 7.4
- 10 mg aliquot each of EDC and sulfo-NHS
- Hydroxylamine solution
- Conjugate diluent
- Fresh DI water
- Microcentrifuge
- Rotator or end-over-end mixer for antibody incubation
- Sonicator

CONJUGATION FLOW CHART



PROCEDURE

Note: *Make sure your antibody is stored in a buffer free of amines groups (e.g. azide, Tris) or protein additives (BSA) before conjugation. See Antibody Buffer Exchange on page 6.*

1. Take out one EDC and sulfo-NHS aliquot package and bring to room temperature (~15 minutes).
2. Thoroughly vortex and sonicate the BioReady™ carboxyl gold particles to disperse and aliquot 1 mL of particles into the microcentrifuge tube provided.

TIME SENSITIVE! Steps 3–4 should be completed within 5 minutes to minimize hydrolysis of the NHS ester in water and ensure conjugation efficiency

3. Pipette 1 mL of fresh DI water to each 10 mg tube of pre-aliquoted EDC and sulfo-NHS (10 mg/mL final concentration). Vortex to ensure all powder is completely dissolved.
4. Add the correct volume of freshly-prepared EDC and sulfo-NHS to the 1 mL aliquot of BioReady™ carboxyl gold according to the table below:

Particles	EDC	NHS
150 nm Gold Nanoshells	8 µL	16 µL
40 nm Gold Spheres	20 µL	40 µL
80 nm Gold Spheres	7 µL	14 µL

5. Vortex solution and incubate for 30 minutes at room temperature (RT) to activate particles.

6. Centrifuge at the following speed and duration for the type of nanoparticle you're working with:

Particles	Speed (RCF)	Duration
150 nm Gold Nanoshells	2000	5 minutes
40 nm Gold Spheres	3800	10 minutes
80 nm Gold Spheres	2000	5 minutes

7. Remove supernatant and resuspend pellet with 1 mL potassium phosphate pH 7.4 reaction buffer. Vortex and sonicate to break up pellet.
8. Repeat steps 6–7 one more time to wash the activated particles with reaction buffer.
9. Add appropriate amount of purified antibody to the particles:

Particles	Ab loading per mL of 20 OD particles
150 nm Gold Nanoshells	30 ug
40 nm Gold Spheres	50 ug
80 nm Gold Spheres	20 ug

10. Vortex to mix and incubate solution for 1 hour at RT on rotator or rocker.
11. After incubation, add 10 µL of quencher (50% w/v hydroxylamine). Incubate at RT for 10 minutes on rocker/rotator.
12. Centrifuge at the appropriate speed and duration for each nanoparticles (see step 6).

13. Remove supernatant and resuspend with 1 mL of **reaction buffer** (wash #1) and vortex/sonicate to mix.
14. Centrifuge and remove supernatant.
15. Resuspend with 1 mL of **reaction buffer** (wash #2) and vortex/sonicate to mix.
16. Centrifuge and remove supernatant. Resuspend with 1 mL of **conjugate diluent**.
17. Keep conjugate at 4°C **(do not freeze).** As an optional step, perform a UV-Vis scan to check for OD and compare the original concentration before conjugation (see *Taking UV-Vis Measurements* on page 36).

Checking Conjugate Performance

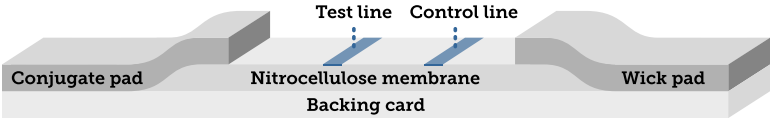
Initial evaluation of conjugate quality can be performed by simply observing the color of the solution after each step. Gold nanoparticles have a distinct visible color that changes when the particles aggregate and simply monitoring the solution color provides a good first assessment of success.

A functionality test can also be performed for the conjugated antibodies to ascertain conjugation efficiency. This is commonly performed in a lateral flow format where a secondary antibody is striped against the species of the antibody-gold conjugate, or with prototype lateral flow strips in the final assay format (with both test and control lines). The following procedure walks you through the assembly and testing process to evaluate your conjugate performance

Note: *This kit does **not** include any strips or materials for the testing of gold conjugate.*

STRIP PREPARATION AND TESTING PROCEDURE

1. **For sandwich assay:** on a nitrocellulose membrane, stripe a test line with capture antibody and a control line with secondary antibody against the host species of the conjugated antibody.
   For competitive assay: stripe antigen as the test line with a similar control line as a sandwich assay (above).
2. Adhere membrane on a backing card.
3. Cut a 20–25 mm wick pad and overlap the wick 3–4 mm onto membrane.
4. Cut 15–20 mm conjugate pad and overlap this onto the other side of the membrane by 2–3 mm.
5. Cut strip to 4 mm or 5 mm width.



6. Prepare a low, medium, and high positive sample with antigen in running buffer.
7. Pipette 6–10 µL of liquid conjugate onto conjugate pad, followed by 15 µL of sample (use running buffer as negative control).
8. Chase the strip with 30 µL of running buffer.
9. Allow strip to run for 15–20 minutes. Strip is ready for readout after the allotted run time.

Alternatively, you can perform the same testing with a half-strip format (membrane and wick pad only; no conjugate pad). This can be done by mixing the liquid conjugate directly with the antigen and running buffer in a well (similar to an ELISA well) or in a 4–6 mL glass culture tube. Then drop the half-strip in the

mixture and allow to run for 15–20 minutes. This method provides a quick assessment of the conjugate binding with antigen without potential variation from adding a conjugate pad.

The presence of the control line indicates proper flow and successful conjugation of antibody onto the nanoparticles. A well-functioning conjugate should also have a clean negative and a dose response for each of the low, medium, and high titrations of the antigen/positive samples.

COMMON ISSUES & SOLUTIONS

Depending on the result of the initial evaluation of conjugate performance, there are a wide variety of further steps that can be taken to address potential issues with the stability of the conjugate, chemical environment (buffers, pH, salts, detergents, etc.), raw materials selection, as well as the assembly and cutting process.

The table on the next page provides a quick summary of issues with possible causes and solutions for further optimization. This list is not meant to be exhaustive but rather a summary of the general strategies for solving commonly-encountered problems while developing an assay. For more details on troubleshooting and optimization steps, please visit our online nanoComposix University course on Lateral Flow Immunoassays at *nanocomposix.com/ncxu-lateral*

Item	Incompatible reaction buffer	EDC/ NHS	Antibody concentration
Absent/weak test & control lines	•	•	•
False positive, NSB			•
Aggregation, poor release from conjugate pad			
Staining of conjugate on membrane			
Solutions	Screen different reaction buffers	Use new/fresh aliquot	Increase or decrease antibody concentration on particles and TL/CL
	Screen conjugation pH	Vary the amount for activation	Increase or decrease antibody incubation time
		For NSB: include a block step or longer blocking time	

Abbreviations:

TL: Test Line

CL: Control Line

NSB: Non-Specific Binding

RB: Running Buffer

Conjugate OD/ concentration	Incompatible buffer chemistry	Unstable conjugate	Incompatible raw materials
•	•	•	•
•	•	•	•
•	•	•	•
•	•		•

Increase or decrease amount of conjugate per strip	*Optimize diluent:* buffers, pH, salts, sugars, detergents, proteins	Screen different membranes, conjugate pads, sample pads
Include a block step or longer blocking time	*Optimize RB:* buffers, pH, salts, sugars, detergents, proteins	
		Block or treat membrane, conjugate pads, sample pads

Once your assay is working (strong control line, positive signal seen on test line), often there is a need to further optimize the performance of assay. Additional experiments that will help you optimize and achieve a more robust, stable, and sensitive conjugate are provided below. These protocols will look at varying antibody loading concentrations, screening reaction buffers, and adding an optional blocking step.

Conjugate Optimization #1: Screen Antibody Loadings

BACKGROUND

The antibody-to-gold ratio is important to optimize in order to prevent non-specific binding and increase assay sensitivity. For 1 mL of 20 OD particles, we recommend evaluating a low, medium, and high concentration of antibodies for this experiment. Higher and smaller loading may be necessary depending on the result from each loading.

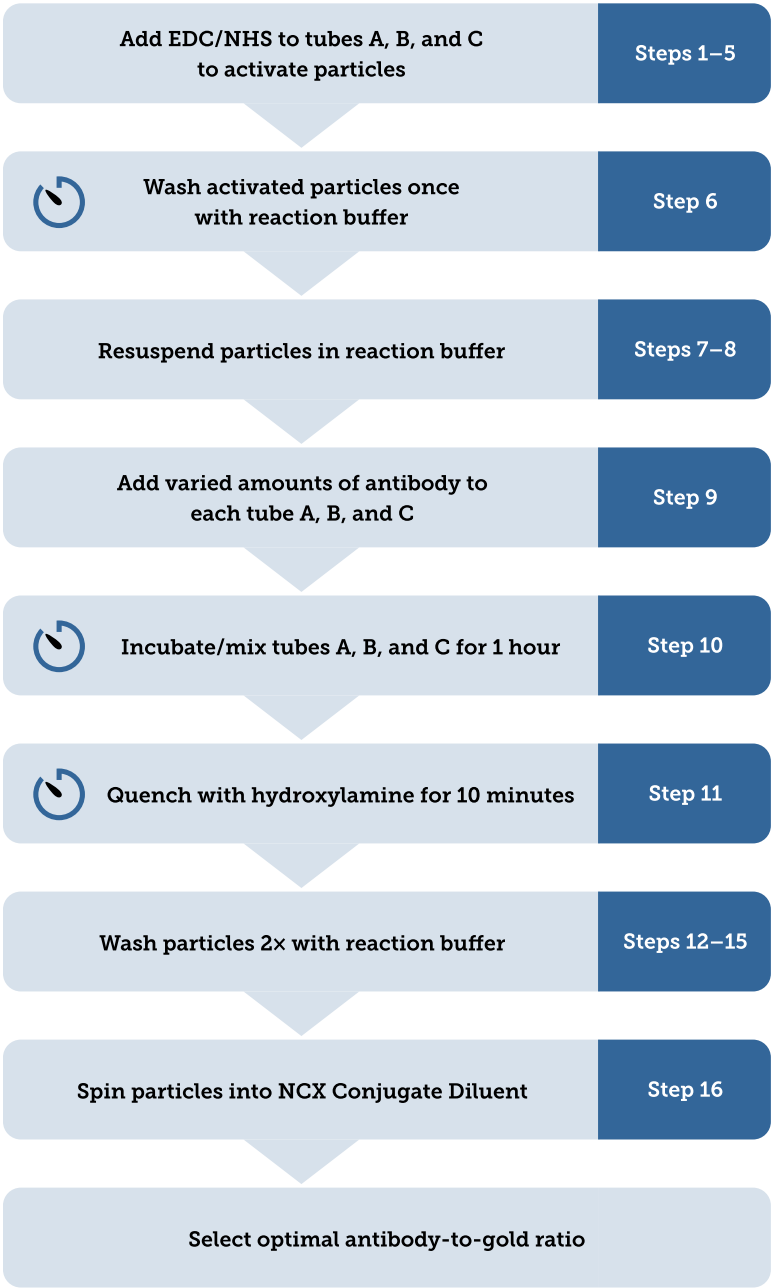
TECHNICAL NOTES

- We begin screening antibody in 2× increments. However, smaller or larger increments may be suitable, depending on the signal intensity of interest. Excess antibody can sometimes negatively impact the conjugation quality.
- For competitive format assays where it is desirable to limit the number of antibodies per particle, we recommend decreasing the antibody screening to 1 µg, 5 µg, and 10 µg of antibody per mL of 20 OD gold nanoparticles and reducing the antibody incubation time to 5–30 minutes.

MATERIALS

- Fresh DI water
- 3 x 1.5 mL LabCon® test tubes
- 3 x 1 mL BioReady™ carboxyl gold particles, 20 OD
- Reaction buffer: potassium phosphate, 0.5% PEG, pH 7.4
- EDC: 10 mg aliquot
- Sulfo-NHS: 10 mg aliquot
- Hydroxylamine solution
- Conjugate diluent
- Microcentrifuge
- Rotator or end-over-end mixer for antibody incubation

ANTIBODY LOADINGS SCREEN: FLOW CHART



ANTIBODY LOADINGS SCREEN: PROTOCOL

1. Take out one EDC and sulfo-NHS aliquot package and bring to room temperature (≥ 15 minutes).
2. Thoroughly vortex and sonicate the BioReady™ carboxyl gold to disperse particles. Aliquot 1 mL of particles into 3 microcentrifuge tubes and label each as A, B, and C.

TIME SENSITIVE! Steps 3–4 should be completed within 5 minutes to minimize hydrolysis of the NHS ester in water and ensure conjugation efficiency

3. Pipette 1 mL of fresh DI water to each 10 mg tube of pre-aliquoted of EDC and sulfo-NHS (10 mg/mL final concentration). Vortex to ensure all powder is completely dissolved.
4. Add the correct volume of freshly-prepared EDC and sulfo-NHS to each tube of 1 mL BioReady™ carboxyl gold according to the table below:

Particles	EDC	NHS
150 nm Gold Nanoshells	8 µL	16 µL
40 nm Gold Spheres	20 µL	40 µL
80 nm Gold Spheres	7 µL	14 µL

Note: *When preparing multiple conditions, both EDC and sulfo-NHS should be added to one sample first before moving on to adding EDC/NHS to another sample. This helps prevent hydrolysis and ensure conjugation efficiency.*

5. Vortex solution and incubate for 30 minutes at room temperature (RT) to activate particles.

6. Centrifuge at the following speed and duration for each type of nanoparticles:

Particles	Speed (RCF)	Duration
150 nm Gold Nanoshells	2000	5 minutes
40 nm Gold Spheres	3800	10 minutes
80 nm Gold Spheres	2000	5 minutes

7. Remove supernatant and resuspend pellet with 1 mL potassium phosphate pH 7.4 reaction buffer. Vortex and sonicate to break up pellet.
8. Repeat steps 6–7 one more time to wash the activated particles with reaction buffer.
9. Add appropriate amount of antibody corresponding to the tube labels:

Nanoparticles	Ab loading per mL of 20 OD particles		
	Tube A	Tube B	Tube C
150 nm Gold Nanoshells	15 ug	30 ug	60 ug
40 nm Gold Spheres	25 ug	50 ug	100 ug
80 nm Gold Spheres	10 ug	20 ug	40 ug

10. Vortex to mix and incubate solution for 1 hour at RT on rotator or rocker.
11. After incubation, add 10 µL of quencher (50% w/v hydroxylamine). Incubate at RT for 10 minutes on rocker/rotator.
12. Centrifuge at the appropriate speed and duration for each nanoparticles (step 6).
13. Remove supernatant and resuspend with 1 mL of **reaction buffer** (wash #1) and vortex/sonicate to mix.

14. Centrifuge and remove supernatant.
15. Resuspend with 1 mL of **reaction buffer** (wash #2) and vortex/sonicate to mix.
16. Centrifuge and remove supernatant. Resuspend with 1mL of **conjugate diluent**.
17. Store conjugate at 4°C. **Do not freeze**.

SELECT OPTIMAL ANTIBODY-TO-GOLD RATIO

Observe the tube and the color of the conjugates after quenching and compare to the "parent" unconjugated material. Only a slight color change should be visible. A lighter blue or the presence of black "specks" is a sign of colloidal instability. For a more precise comparison of color, compare the UV-Vis spectra before and after conjugation (see *Taking UV-Vis Measurements* on page 36). Alternatively, run the conjugate on a test strip and measure the performance (see page 16). Select the antibody loading which resulted in the best colloidal stability, lowest non-specific binding (NSB), and highest positive signal intensity. In the case that all three antibody loadings perform equally, we recommend moving forward to the next optimization step with the lowest antibody loading. If one of the antibody loadings generates superior performance but there is still non-specific binding present, a second antibody screen in smaller increments is recommended.

If optimizing antibody loading does not eliminate non-specific binding, other parameters may be optimized to reduce non-specific binding which include a blocking step, buffer optimization, conjugate pad pre-treatments, and raw materials selection (see page 17). Additional information on all these assay optimizations can be found in the **Lateral Flow Assay Development Handbook** at *nanocomposix.com/bioready*.

Conjugate Optimization #2: Reaction Buffers Screen

BACKGROUND

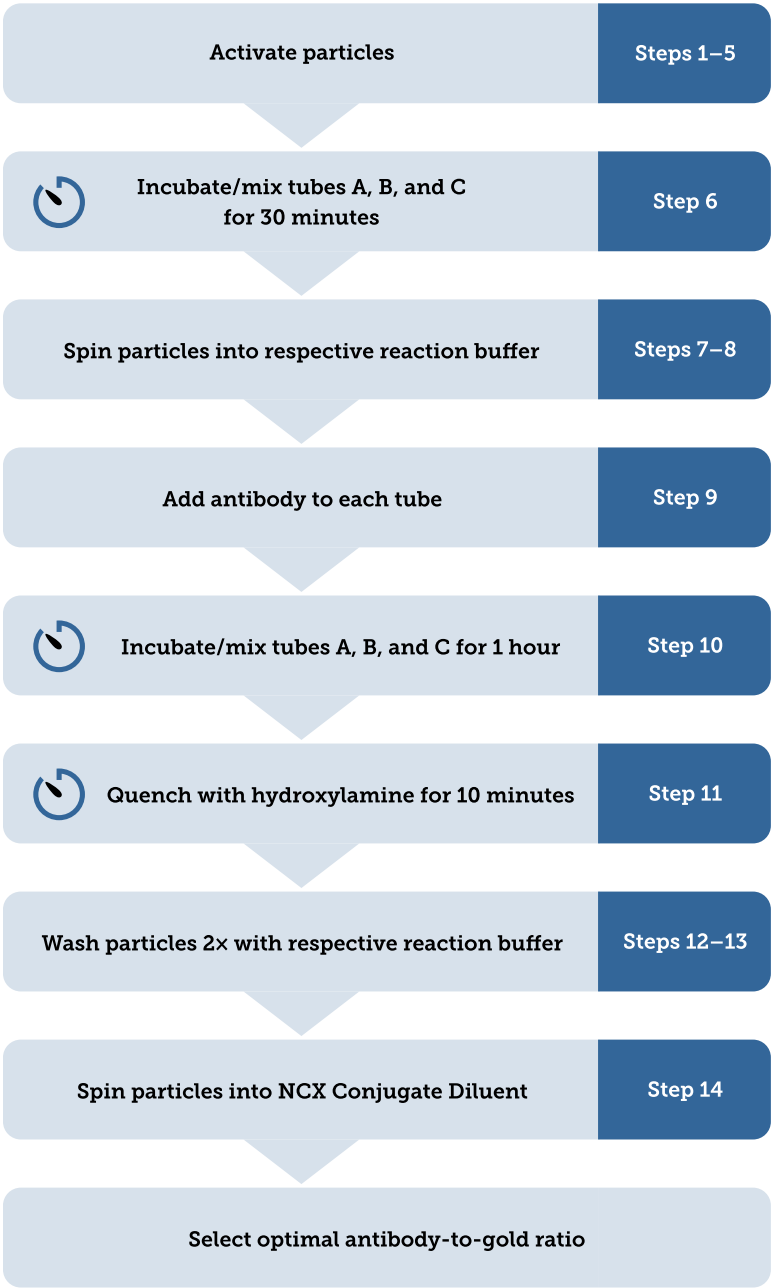
Due to the uniqueness of each protein, different reaction buffer formulations can affect how the proteins behave in solution. The protein behavior will determine which lysine residues are presented for amide bond formation with the sulfo-NHS ester on the activated particles. While activation of NHS esters is most efficient at pH 5, conjugation of sulfo-NHS esters to amine groups on proteins is most efficient at pH 7–8. Upon addition of EDC and sulfo-NHS, the gold nanoparticle solution will drop to pH 5. After activation, it is important to transfer the activated particles to a neutral pH for amide bond formation with the protein of interest.

This reaction buffer screen will test three different buffer formulations that are most commonly used at nanoComposix during lateral flow assay development.

MATERIALS

- DI water
- 3 x 1.5 mL LabCon® test tubes
- 3 x 1 mL aliquot BioReady™ carboxyl gold particles, 20 OD
- Reaction buffers:
  A. 5 mM potassium phosphate, 0.5% PEG 20 kDa, pH 7.4
  B. 5 mM sodium phosphate, 0.5% PEG 20 kDa, pH 7.4
  C. 0.5X PBS, 25% PEG 20 kDa, pH 7.4 (50× concentrated stock) – *dilute* to 1X (0.01X PBS, 0.5% PEG 20 kDa) by adding 200 µL of 50X stock into 10 mL fresh DI water. Check pH to ensure it falls between 7.0–7.6
- EDC: 10 mg aliquot
- Sulfo-NHS: 10 mg aliquot
- Hydroxylamine solution
- Conjugate diluent
- Microcentrifuge
- Rotator or end-over-end mixer for antibody incubation

REACTION BUFFERS SCREEN: FLOW CHART



REACTION BUFFERS SCREEN: PROTOCOL

1. Take out one EDC and sulfo-NHS aliquot package and bring to room temperature.
2. Thoroughly vortex and sonicate the BioReady™ carboxyl gold to thoroughly disperse particles. Aliquot 1 mL of particles into 3 microcentrifuge tubes and label each as A, B, and C.

TIME SENSITIVE! Steps 3–4 should be completed within 5 minutes to minimize hydrolysis of the NHS ester in water and ensure conjugation efficiency

3. Pipette 1 mL of fresh diH_2O to each 10 mg of pre-aliquoted tube of EDC and NHS (10 mg/mL final concentration).
4. Add freshly-prepared EDC and NHS to each of the 1 mL BioReady™ carboxyl gold:

Particles	EDC	NHS
150 nm Gold Nanoshells	8 µL	16 µL
40 nm Gold Spheres	20 µL	40 µL
80 nm Gold Spheres	7 µL	14 µL

Note: *When preparing multiple conditions, both EDC and NHS should be added to one sample first before moving on to adding EDC/NHS in another sample. This helps prevent hydrolysis and ensure conjugation efficiency.*

5. Vortex solution and incubate for 30 minutes at room temperature (RT) to activate particles.

6. Centrifuge at the following speed and duration for each type of nanoparticles:

Particles	Speed (RCF)	Duration
150 nm Gold Nanoshells	2000	5 minutes
40 nm Gold Spheres	3800	10 minutes
80 nm Gold Spheres	2000	5 minutes

7. Remove supernatant and resuspend each pelleted nanoparticle with 1 mL of **reaction buffer** that corresponds to the tube label:
    A. 5 mM potassium phosphate, 0.5% PEG 20 kDa, pH 7.4
    B. 5 mM sodium phosphate, 0.5% PEG 20 kDa, pH 7.4
    C. 0.01X PBS, 0.5% PEG 20 kDa, pH 7.4*
    ***Freshly diluted from 50× stock by user**

    Note: *Observe any differences in the pellet between each tube; plating on the inside of the tube may result in an unstable conjugate.*

8. Vortex and sonicate to fully resuspend particles.
9. Repeat steps 6–8 one more time to wash the activated particles with each respective reaction buffer.
10. Add appropriate amount of antibody to each 1 mL tube:

Particles	Antibody loading per mL of 20 OD particles
150 nm Gold Nanoshells	30 µg or selected concentration from Optimization #1
40 nm Gold Spheres	50 µg or selected concentration from Optimization #1
80 nm Gold Spheres	20 µg or selected concentration from Optimization #1

11. Vortex to mix and incubate solution for 1 hour at RT on rotator or rocker.
12. After incubation, add 10 µL of quencher (50% w/v hydroxylamine). Incubate at RT for 10 minutes on rocker/rotator.
13. Centrifuge at the appropriate speed and duration for each nanoparticles (step 6).
14. Remove supernatant and resuspend with 1 mL of **each reaction buffer** (wash #1) and vortex/sonicate to mix.
15. Centrifuge and remove supernatant.
16. Resuspend with 1 mL of **each reaction buffer** (wash #2) and vortex/sonicate to mix.
17. Centrifuge and remove supernatant. Resuspend with 1mL of **conjugate diluent.**
18. Store conjugate at 4°C. **Do not freeze**.

SELECT OPTIMAL REACTION BUFFER FORMULATION

Observe the visible color of the conjugate and compare to the stock nanoshell color. Only a slight color change should be visible. For a more precise comparison of color, compare the UV-Vis spectra before and after conjugation (see *Taking UV-Vis Measurements* on page 36). If using lateral flow as a functional readout, run the conjugate on a test strip and measure the performance (see page 16). Note any particle aggregation at the sample pad/nitrocellulose interface, non-specific binding, and the relative signal intensity strength of the positive signal. Select the reaction buffer that resulted in the best colloidal stability, lowest non-specific binding, and highest positive signal intensity. If all three reaction buffers perform equally, we recommend moving forward to the next optimization step with 5 mM potassium phosphate 0.5% PEG 20 kDa, pH 7.4.

Common Pitfalls to Avoid

It is important to understand that each antibody is unique and may exhibit different performance when conjugated under different conditions compared to other proteins. There is no one-size-fits-all conjugation method and each antibody or assay may need to be optimized individually for best results. However, there are several areas that should be controlled across different conjugation experiments in order to ensure reproducible and robust conjugate performance:

Mis-handling EDC & NHS: The EDC and sulfo-NHS aliquots provided are intended for single use. Be sure to let EDC equilibrate to room temperature (~20 minutes) prior to the opening of the EDC. Use the EDC as soon as possible after resuspension and use a fresh aliquot of EDC for each coupling reaction.

Deviations from protocol: For conjugation to BioReady™ gold nanoparticles, adding excess activation reagents, protein, or detergents can compromise the performance of the antibody. We recommend following the guidelines in this protocol before trying other conditions.

Inappropriate tubes: Many plastic tubes contain plasticizers or residual mold-release lubricant that can negatively impact the covalent conjugation chemistry used in this protocol. Use the tubes provided for initial conjugation work and then verify that your tube brand is an acceptable substitute before switching to a new type of tube. We recommend LabCon® 1.5 mL, 15 mL and 50 mL volume tubes.

Long periods at room temperature: While covalent conjugates are much more robust than conjugates prepared using passive conjugation methods, avoid long centrifuge times (> 30 min) that can inadvertently heat the conjugate.

Unpurified antibody: For covalent conjugation it is critical to purify the antibody or protein of interest away from any primary amines. A purification column is included in the kit to ensure that your antibody is ready to use (see *Antibody Buffer Exchange* section on page 6).

Conjugate Stability & Performance

This kit will help you develop an optimized conjugate with maximized performance. During the optimization process you will have to assess which conditions yield the best result. The best and most relevant method of determining conjugate performance is with functional testing (see page 16). However, initial evaluation of conjugate quality can be performed by simply observing the color of the solution after each step. Gold nanoparticles have a distinct visible color (see Figure 1) that changes when the particles aggregate and simply monitoring the solution color provides a good first assessment of success. If you have access to a UV-Vis spectrophotometer, the "color" of the conjugates can be more precisely tracked. In the following sections, we describe how both spectroscopy and lateral flow tests can assess conjugate quality.

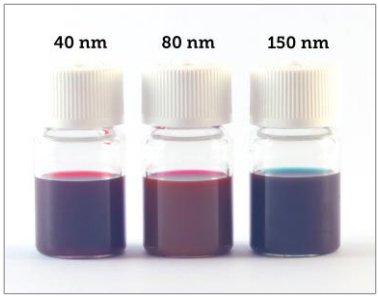


Figure 1: Photo of 40 nm Gold Spheres, 80 nm Gold Spheres, and 150 nm Gold Nanoshells, all at 20 OD.

ANALYTICAL QC CHECK: UV-VIS SPECTROSCOPY FOR MONITORING CONJUGATE STABILITY

A simple method for evaluating conjugate stability is to measure and compare the UV-Vis spectra before and after conjugation. When a protein binds to a nanoparticle surface, there is a change in the local refractive index which can be observed in the UV-Vis spectrum as a small shift to longer wavelengths. Figure 2 shows a successful conjugation on 150 nm gold nanoshells where the peak resonance has shifted to the right by about 3 nm (red-shifted). This is due to the effect of the conjugated protein on the plasmon resonance of the gold nanoshell.

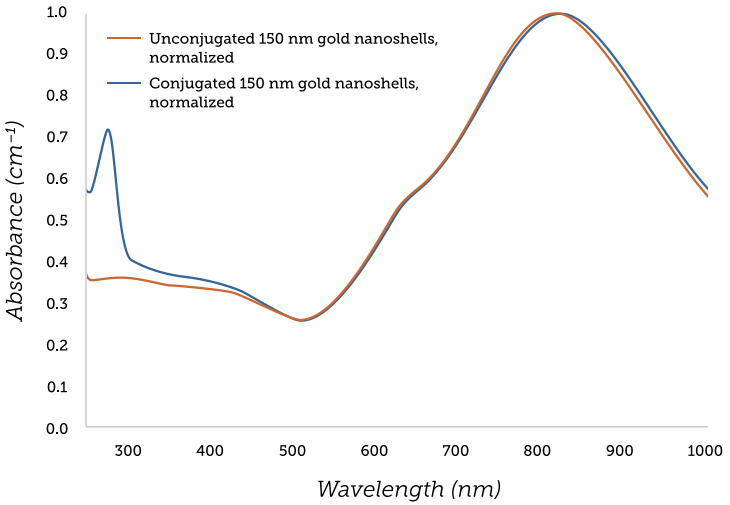


Figure 2. Normalized UV-Vis spectra of 150 nm gold nanoshells before and after conjugation to antibody.

If the particles are not stable after a conjugation, there will be change in the color and corresponding UV-Visible spectrum of the particle solution. The peak absorbance will decrease and the peak will broaden. Figure 3 shows example spectra of a particle solution that has been partially destabilized. Further investigation of the conditions that led to the destabilization should be

investigated to try to obtain conjugates that do not exhibit this change in the optical properties.

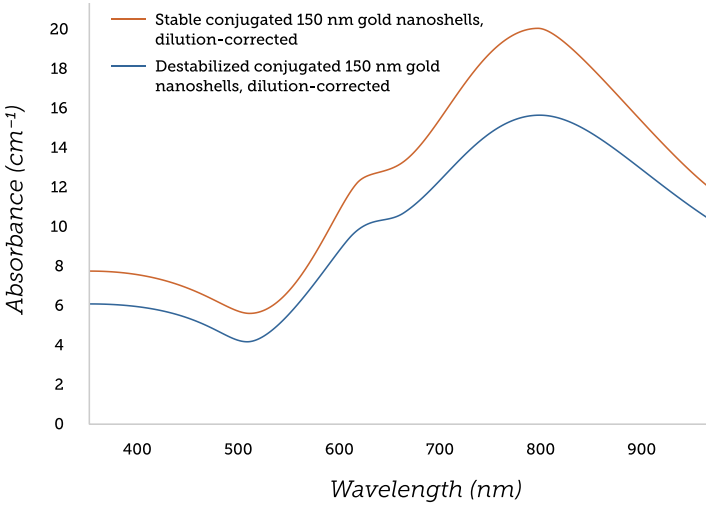


Figure 3. Dilution-corrected UV-Vis spectra of 150 nm gold nanoshells, stable conjugate compared to destabilized conjugate.

TAKING UV-VIS MEASUREMENTS

During most of the conjugation steps in this protocol, the resulting conjugates will be concentrated, typically to ~20 optical density units (OD). This is too concentrated for most UV-Vis instruments. We recommend finding the lowest volume that your UV-Vis instrument will measure and then diluting sufficient concentrated conjugate to obtain a solution with an OD between 0.5 and 1.0. This will allow you to use only the minimum amount of material required to generate reliable data. A typical dilution for nanoComposix conjugates is a 20× dilution (e.g., 50 µL of 20 OD conjugated gold nanoshells + 950 µL of DI H_2O). Correct for the dilution when calculating OD.

Further Conjugate Optimization

SAMPLE MATRIX

Initial assay optimization is often performed in buffer solutions that are free of any potential contaminants or other components that may lead to non-specific binding. Testing in buffer allows for a quick and clean evaluation of the antibody-antigen binding characteristics. However, these conditions are unlikely to be the final optimized format of the assay. Another set of optimizations will be necessary after transitioning to the target matrix (e.g. serum, saliva, urine, whole blood etc.). Sample matrices such as saliva, serum, whole blood, urine, etc. contain extraneous material that can bind to your conjugate non-specifically, which can result in false-negative or false-positive results. When transitioning to a more complex sample type, it is recommended to re-screen conditions such as conjugate diluent formulation, running buffer chemistry, necessity of a blocking step, and raw materials re-selection.

INCORPORATION OF A BLOCKING STEP

Provided with this kit is an NCX Blocking Buffer with a formulation of 4 mM sodium borate 1% BSA at pH 8.2. Blocking a conjugate can often decrease non-specific binding via the addition of non-specific proteins such as bovine serum albumin (BSA) or casein protein. NanoComposix standard protocols include a 1 hour blocking step with the supplied blocking buffer. Blocking is typically performed immediately after antibody incubation or quenching step.

Conjugate Blocking Protocol: After the antibody incubation or quenching step, sonicate and aliquot 0.5mL of the conjugate into a separate tube. Spin conjugate and remove ~500 µL of supernatant and resuspend with ~500 µL NCX Blocking Buffer.

Incubate/mix for 1 hour. Centrifuge to remove supernatant and perform 2 washes with the reaction buffer (see *Conjugation Procedure* on page 14). Resuspend in the NCX Conjugate Diluent.

Compare performance of blocked conjugate to unblocked conjugate. If the blocking step improves performance, it is recommended to investigate 30 minutes, 1 hour, 2 hour, and overnight blocking times at varying concentrations of protein (1-10%). Alternatively, different types of blocking proteins (BSA, casein, gelatin) and detergents can also be evaluated if necessary. If the blocking step did **not** demonstrate an improvement in conjugate performance or stability, we recommend that you do not include a blocking step.

Blocking can also be incorporated into other aspects of the assay such as membrane, conjugate pad, and in buffer formulations.

REACTION BUFFER, ANTIBODY LOADING, AND INCUBATION

The steps in this kit are the recommended strategy for a few initial optimizations of a stable conjugate. However, further optimization of each of these parameters may yield additional improvements. The reaction buffer formulation and blocking formulations can be varied by salt concentration or by addition of other stabilizing agents. Antibody loading and incubation times may require further tuning up or down. To order more BioReady™ materials to perform additional experiments, visit *nanocomposix.com/bioready*.

CONJUGATE DILUENT FORMULATION OPTIMIZATIONS

Each antibody and each assay system will require a different optimal storage/diluent buffer. The components of the conjugate diluent may be varied or fine-tuned to optimize stability and shelf life of your conjugate. Protein additives such as BSA or casein

should be investigated as these components can sometimes eliminate non-specific binding or assist with flow properties. Screening different buffering salts, sugars, pH conditions, and surfactants/detergents is also an integral step in formulating an optimal buffer for your conjugate. More information on these can be found at *nanocomposix.com/ncxu-lateral*

PARTICLE SELECTION

BioReady™ 40 and 80 nm carboxyl Gold Spheres are an alternative to the 150 nm carboxyl Gold Nanoshells. For lateral flow assays, the 40 and 80 nm particles often require lower OD-loading to achieve their respective maximum sensitivity, reducing the cost per strip. However, using these smaller particle sizes will typically result in a lower maximum possible sensitivity compared to using gold nanoshells.

A small percentage of antibodies are not suitable for covalent conjugation and will not result in a colloidally stable conjugate. For these cases, passive adsorption on "bare" particle surfaces (such as BioReady™ carbonate or citrate) should be investigated. The 40 and 80 nm sizes are available with a carbonate surface, which is specifically designed for passive adsorption. For some antibodies passive adsorption works very well, and it is a good idea to try both covalent and passive adsorption during development.

Troubleshooting & FAQ

My antibody is already purified. Do I really need to purify it again?

Many antibodies are purified using protein A or Protein G affinity columns. During this purification process, Tris is often used to elute the antibody off the columns and may be present in varying amounts in the final antibody solution. We strongly encourage purification of all antibodies to remove any possible unknown primary amines which interfere with covalent conjugation (see *Antibody Buffer Exchange* section on page 6).

Do you recommend using MES Buffer for activation?

MES buffer is often used in activation protocols for latex and fluorescent beads to adjust the reaction to pH 5. NanoComposix BioReady™ materials are provided in a mildly buffered aqueous solution that adjusts to pH 5 upon addition of EDC and sulfo-NHS. It is not necessary to activate BioReady™ materials in MES buffer.

Can I just add an excess of antibody rather than performing an optimization?

In many cases, too high or too low of an antibody addition can lead to non-specific binding and a decrease in sensitivity. This is why it is important to empirically determine the mass of antibody addition.

I don't see any trends in my antibody loading titration. What happened?

The antibody loading ratios screened in this kit may not be optimum for your antibody or application. You may need to evaluate antibody loading ratios above or below the values investigated within this kit.

After I diluted my 50× reaction buffer, the pH was not within the acceptable range of 7.0–7.6. What happened?

This reaction buffer has an intentionally low buffering capacity. *If* diluted with water that has been exposed to the atmosphere for prolonged periods, the pH may no longer be neutral. Prepare a new dilution with fresh DI water.

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 solubilized just prior to conjugation. Sulfo-NHS should always be stored between 4–8°C, and EDC should be stored at –20°C or between 4–8°C. *It is important to keep the reagents desiccated and allow them to come to room temperature prior to opening the bottles to avoid condensation from the atmosphere as both EDC and sulfo-NHS are moisture-sensitive.*

What other methods of functional testing can I perform if I do not have access to lateral flow strips?

Functional testing should closely mimic the end-use of your conjugate for accurate performance validation. For example: if the application of the conjugate is for a colorimetric ELISA, it is recommended that you coat wells of a 96-well plate with the appropriate antibody or protein to bind your conjugate. Applications may vary. If you have further questions regarding functional testing, please contact us at *info@nanocomposix.com*, or call +1 (858) 565-4227.

Are the conjugates salt stable?

Unconjugated gold nanoparticles are not salt stable, but after successful conjugation, conjugates are generally stable in 1X PBS, and some customers have observed stability in much higher salt concentrations. If your application requires a high-salt

environment, we recommend preparing your conjugate at different salt concentrations, and monitoring the conjugate stability by UV-Vis. A broadening in the peak and drop in optical density are indicative of instability.

Can I add more EDC and Sulfo-NHS to achieve better binding efficiency?

The amounts of EDC and sulfo-NHS in these protocols have been carefully determined to generate the best performance. A larger excess may cause the particles to aggregate, resulting in poor conjugation.

I am having trouble resuspending my conjugate after centrifugation.

The particles can sometimes form hard pellets that are difficult to resuspend. A bath sonicator is highly recommended for resuspension, but vigorous vortexing (~30 seconds on high setting) is usually sufficient to resuspend the pellet. Inspect the conjugate solution after resuspension to ensure there are no visible particulates.

Are your particles tolerant to detergents like Tween?

Stable conjugates optimized with this procedure are usually stable with most detergents and polymers commonly used in bioconjugation applications.

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 half as dense as a solid 150 nm gold particle and flow easily through a nitrocellulose membrane. It is important to note that at the same optical density, there are about 30× fewer nanoshells by particle number, so conjugate volumes will need to be adjusted appropriately to

maximize binding events. As a starting point to boost sensitivity, we recommend doubling either OD or conjugate volume per strip relative to the amount of 40 nm gold you would typically use. A full sweep of conjugate loading per strip is recommended.

Do you perform custom conjugations or assay development services?

Yes! Please contact us regarding our custom capabilities and collaboration efforts. We can be reached at *info@nanocomposix.com*, or +1 (858) 565-4227.

I am having trouble interpreting my results.

For help interpreting your results, you can email us at *info@nanocomposix.com*, or call +1 (858) 565-4227.

Can I use your BioReady™ materials for commercial applications?

Absolutely. Please contact us to discuss licensing and any applicable regulatory or compliance requirements for your intended commercial use. We can be reached at *info@nanocomposix.com*, or +1 (858) 565-4227.

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. We specialize in full-service lateral flow development and are excited to help our customers perform early-stage R&D or bring products to market.

This kit is intended for optimization of covalent conjugation of antibodies, Fab fragments, or other proteins to nanoComposix BioReady™ carboxyl gold nanoparticles. Every protein is unique, so the conjugation procedure needs to be customized to achieve optimal stability and performance.

For research use only.

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Accelerating Nanotechnology Commercialization

This kit is intended for optimization of covalent conjugation of antibodies, Fab fragments, or other proteins to nanoComposix BioReady™ carboxyl gold nanoparticles. Every protein is unique, so the conjugation procedure needs to be customized to achieve optimal stability and performance.

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