Covalent Binding for Robust, Reliable and Reproducible Lateral Flow Detector Particles

Description

Lateral flow assays are rapid, self-contained diagnostic tests that can detect a variety of analytes. The test is inexpensive, easily portable, stored at ambient temperature, and has a long shelf life. The assay provides diagnostic results without sample processing or additional equipment, making this format ideal for point-of-care and field-based diagnostics. A key component of every lateral flow test is the detector particles that are coated with a molecular recognition element (typically an antibody). A common type of detector particles used in lateral flow assays are gold nanoparticles which have very large absorption cross sections making them easy to visualize when they bind to a test line (see Figure 1).

Figure 1. Many commercial pregnancy tests use gold nanoparticles for detection. The first red line indicates a positive result. The second red line is a positive control to demonstrate that the assay is working.

To fabricate gold conjugate detector particles, the molecular recognition element must be bound to the surface of the gold particle. One method of creating these antibody-particle conjugates is to simply mix the gold nanoparticles and antibody together and allow the antibody to bind to the gold nanoparticle surface through physisorption. While this is often successful, each physisorption conjugation must be optimized with respect to salt concentration, pH, and antibody/particle ratio. In some cases, the conjugation of the antibody to the particle surface is weak and the antibodies can dissociate from the surface, potentially destabilizing the particle or reducing the particle's binding affinity for analytes. An alternative method for binding antibodies to the particle surface is through the use of covalent chemistry. Covalent binding offers a number of advantages including:

- Increased conjugate stability in a wider range of sample matrices
- Greater control over the particle/antibody ratio
- Reduction in the number of conjugation reactions necessary to find optimal antibody pairs

This white paper provides additional information on how to form robust and reliable antibody conjugates through the use of covalent binding.

Particles for Covalent Binding

NanoComposix offers three different BioReady nanoparticles for covalent binding: 40 nm diameter gold nanospheres, 80 nm diameter gold nanospheres and 150 nm diameter gold nanoshells. The particles are functionalized with lipoic-PEG-acid (for nanospheres) or lipoic acid (for nanoshells) to yield a robust carboxyl surface for covalent binding to free amines on the selected targeting agent. Covalent amide bonds between the carboxyl and free amines are achieved through an EDC/Sulfo-NHS intermediary (**Figure 2**). 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 accessible lysine groups.

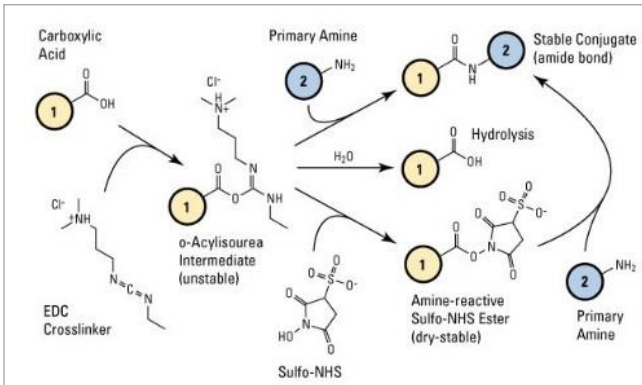

Figure 2: EDC-NHS reaction scheme – from Thermo-Fisher.

Conjugation Protocol: Step 1: Antibody Purification

Prior to conjugation, it is critical that the antibody solution does not contain additional free amines in the storage buffer as the free amines will compete with binding sites on the nanoparticle. Free amines including those found in tris buffer or the preservative sodium azide must be removed and the antibody must be transferred to a suitable amine-free buffer. One common way of performing this wash step is to use spin columns (**Figure 3**). Additionally, any additional stabilizing proteins in the antibody solution must also be removed. Protein A or other affinity columns can be employed to isolate the antibody from other proteins. Protein purification should be performed first since the elution of the antibody from affinity columns often involves the use of amine containing buffers. A subsequent purification of the antibody into an amine-free buffer is then required. Steps involved in purification of antibodies are detailed below.

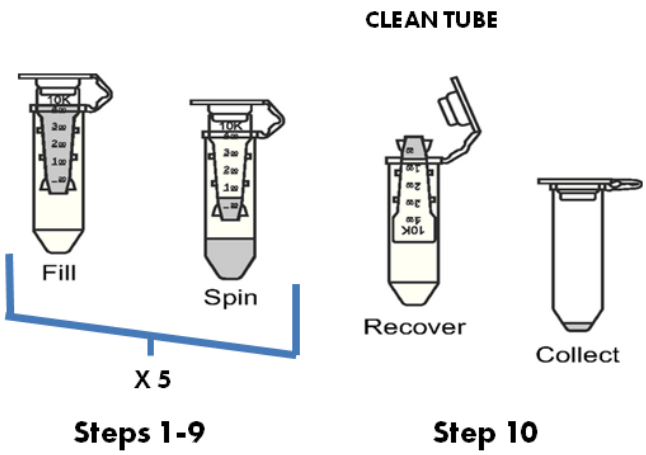

Figure 3: Method to purify proteins with spin columns.

MATERIALS

- Millipore Amicon Ultra 0.5 mL 10K filters for protein purification and concentration (Catalog# UFC501096)
- 2 mL Microcentrifuge tubes to hold filters
- Microcentrifuge
- Antibody to be purified, (e.g. 0.1 to 2 mg Ab per filter)
- Purification buffer (e.g. 10 mM potassium phosphate or other amine-free buffer)
- BCA assay kit and plate reader or UV-vis spectrophotometer for protein quantification

PURIFICATION PROTOCOL

1. Place filter inside microcentrifuge tube.
2. Add 450 µL of the purification buffer and centrifuge 5 minutes at 13.8k RCF to pre-rinse the filter.
3. Dispose of the filtrate at the bottom of the tube.
4. Aliquot antibody solution into filter and close cap. The filter can hold up to 500 µL. If the volume to purify exceeds this capacity, centrifuge to concentrate and add more unpurified antibody before continuing with the wash steps. If the starting antibody volume is minimal, add buffer up to ~450 µL total volume.
5. Centrifuge for 5 minutes at 13.8k RCF to concentrate.
6. Remove the filter containing the concentrated antibody from the microcentrifuge tube. Remove the filtrate solution from the bottom of the microcentrifuge tube.

   NOTE: The filtrate can be retained from all wash steps in a clean container. If the yield is particularly low due to punctured filter, the antibody may be reclaimed from the retained filtrate.

7. Place the filter containing the concentrated antibody back into the tube and add 350 µL of purification buffer to the filter.
8. Centrifuge for 5 minutes at 13.8k RCF to wash/concentrate.
9. Repeat washing procedure (steps 5-7 above) an additional four times using 350 µL of additional purification buffer for a total of five washes.
10. After the final wash, turn device upside down in a *new, clean* 2 mL microcentrifuge tube and cut off the cap. Centrifuge for 5 minutes at 1k RCF to collect purified and concentrated antibody. For optimal recovery, perform the reverse spin immediately.
11. Bring the antibody solution to a final concentration of ≥1 mg/mL for storage. After purification, the typical recommended storage is to aliquot and store purified antibodies ≥1 mg/mL based on the antibody supplier's certificate of analysis. In general, freeze/thaw cycles should be avoided. Refer to the antibody supplier's instructions for the recommended storage and handling procedures.

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DETERMINING FINAL PROTEIN CONCENTRATION

Now that you have purified your antibody, it is important to determine the final protein concentration. This can be done by using a method based on the absorbance of the solution at 280 nm in a spectrophotometer (A_{280} method) or the bicinchoninic acid (BCA) assay.

MAXIMIZING SAMPLE RECOVERY

Low sample recovery in the concentrate may be due to adsorptive losses, over-concentration, or passage of the sample through the membrane. To maximize sample recovery, ensure that the pipette tip does not puncture the membrane filter. Adsorptive losses depend upon the solute concentration, hydrophobicity, temperature, time of contact with the filter device surfaces, sample composition and pH. To minimize losses, remove concentrated samples immediately after centrifugation. If the starting sample concentration is high, monitor the centrifugation process in order to avoid over-concentration of the sample. Over-concentration can lead to precipitation and potential sample loss. If the sample appears to be passing through the membrane, choose a lower nominal molecular weight limit (NMWL) Amicon Ultra-0.5 filter unit. After collecting concentrated/purified antibody, the inside of the filter can be rinsed a few times with a small volume of purification buffer to reclaim any of the antibody that may be on the filter membrane.

Conjugation Protocol: Step 2: Antibody Conjugation

Antibodies can be conjugated to the terminal carboxy functional groups on the surface of the nanoparticle through carbodiimide crosslinker chemistry (EDC). EDC reacts with the carboxylic acid groups to form an active-ester intermediate. The addition of Sulfo-NHS increases solubility and stability of the intermediate, which reacts with the amine group of the antibody to form a stable amide bond between the antibody and nanoparticle. The method below describes the steps for antibody conjugation of carboxyl functionalized nanoparticles.

MATERIALS REQUIRED

- 40 nm diameter BioReady carboxyl gold nanoparticles
- Purified antibody with no additional free amines
- Reaction buffer (5 mM potassium phosphate, 0.5% 20K MW PEG, pH 7.4)
- EDC
- Sulfo-NHS
- Quencher (50% (w/v) hydroxylamine)
- Conjugate diluent (0.1X PBS, 0.5% BSA)
- Centrifuge
- Standard microcentrifuge tubes (with no specialized treatments or residual plasticizer)
- Vortex
- Rotator

CONJUGATION PROTOCOL

The provided conjugation strategy is described for 1 mL of 40 nm diameter carboxyl gold nanoparticles at OD 20 that will result in 1 mL of antibody-gold conjugate at OD 20. For larger or smaller volumes, scale proportionately or follow the specific conjugation protocol provided with each BioReady carboxyl variant.

IMPORTANT: Steps 1-6 should be completed immediately after solubilizing EDC/Sulfo-NHS to minimize hydrolysis of the Sulfo-NHS ester in water and enhance the efficacy of conjugation.

1. Prepare EDC and Sulfo-NHS at 10 mg/mL in H_2O immediately before conjugation steps.

TIP: Ensure the EDC and Sulfo-NHS reagents are at room temperature before opening vials. Weigh out approximately 1-10 mg EDC and Sulfo-NHS each in individual microcentrifuge tubes. Just prior to conjugation, dissolve in the appropriate volume of H_2O to bring the concentration to 10 mg/mL.

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Example: Mass of EDC = 2.38 mg, add 238 µL H_2O
Mass of Sulfo-NHS = 6.14 mg, add 614 µL H_2O

2. Add 200 µg of EDC (20 µL freshly prepared EDC at 10 mg/mL in H_2O) and 400 µg of Sulfo-NHS (40µL of freshly prepared Sulfo-NHS at 10 mg/mL in H_2O) to 1 mL of 40 nm carboxyl gold nanoparticles.
3. Vortex solution and incubate at room temperature for 30 minutes while rotating.
4. Centrifuge at 3600 RCF for 10 minutes.
5. Carefully remove the supernatant to remove any excess EDC/Sulfo-NHS and re-suspend in 1 mL of reaction buffer. Vortex and/or sonicate to fully re-suspend particles.
6. Add antibody and vortex solution.
7. Incubate at room temperature for 2 hours while rotating. Note that shorter or longer incubation times may improve the efficacy of conjugation.
8. After incubation, add 10 µL of Quencher to deactivate any remaining active NHS-esters. Vortex and incubate at room temperature for 10 minutes while rotating.
9. Centrifuge at 3600 RCF for 10 minutes. Carefully remove supernatant and re-suspend in 1 mL of reaction buffer. Vortex and/or sonicate to fully re-suspend conjugate.
10. Repeat centrifugation and re-suspension to remove any excess antibody.
11. Centrifuge again at 3600 RCF for 10 minutes, remove the supernatant and bring the volume up to 1 mL with Conjugate Diluent. Vortex and/or sonicate to fully re-suspend conjugate.
12. Store conjugate at 4°C**.** Do not freeze**.**

ADDITIONAL TIPS FOR OPTIMIZING THE CONJUGATE

It is important to note that the optimal conjugation procedures are antibody dependent and the optimization techniques will differ between antibodies. The conjugates should be stable in solution and have efficient and specific binding to the antigen. The following tips can improve the efficacy of the conjugate:

- The activation of the carboxylic acid group with EDC and Sulfo-NHS is most efficient at pH 5.
- The pH of the reaction of primary amines on the antibody with the activated carboxyl groups is most efficient at pH 7-7.5. Performing the reaction at a higher pH drastically reduces the half-life of the NHS-ester intermediate.
- The concentration of the antibody during conjugation and the incubation time between the antibody and the nanoparticles can be adjusted to determine the optimal conditions.
- The conjugate diluent components can be adjusted to determine the optimal buffer molarity, pH, blocking agents, polymers and surfactants.

Conjugation Protocol: Step 3: Characterize the Conjugates

The characterization of conjugates is critical to ensure that the antibody is bound to the surface of the particle and that the conjugate is stable. UV-Vis spectroscopy is one tool that can be utilized to evaluate the stability by looking at the plasmon resonance absorption. A slight shift in resonance peak position of the nanoparticle before and after conjugation indicates that the antibody has been successfully conjugated to the surface. If the conjugate is aggregated, the peak shifts and becomes broader **(Figure 4)**. Dynamic light scattering is another tool that can be used to screen for small amounts of aggregation by measuring the hydrodynamic size and the polydispersity index. Lateral flow test strips are also an effective way of evaluating conjugate performance, while also being a common application for such conjugates. The lateral flow test strip can contain reagents that are specific for the antibody conjugated to the

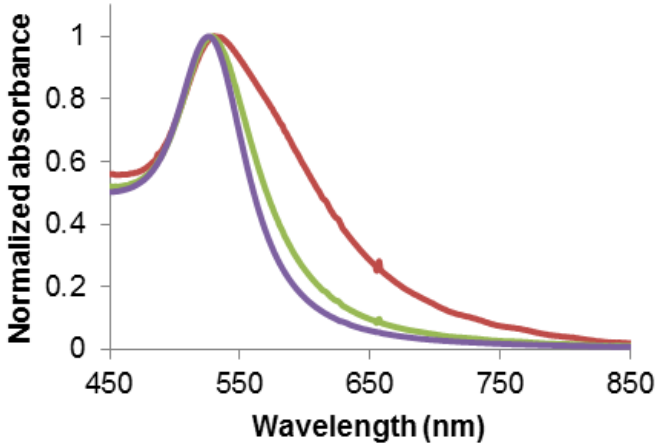

Figure 4. UV-Vis spectra of carboxyl terminated 40 nm Au (blue), protein conjugated gold (green) and aggregated 40 nm Au (red).

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nanoparticle and provides a fast and simple method for determining whether an antibody was successfully conjugated and still retains function.

LOT SIZE AND PRICE PER STRIP

BioReady carboxyl particles are fabricated within nanoComposix's ISO 13485 compliant quality system ensuring high lot-to-lot consistency of particle properties. Lot sizes as large as 400,000 OD-mLs (equivalent to 400 L of 1 OD gold) can generate ~1,000,000 strips from a single lot. In a supply contract, customers can reserve particular lots to allow for sampling from the same lot for up to a year, reducing any downtime due to re-optimization when switching to a new lot. The large lot manufacturing also allows us to offer our extensively characterized product at very competitive pricing. When comparing vendor pricing, it is important to adjust for prices that are supplied for different volumes and concentrations. To normalize pricing, we typically talk about cost in terms of OD-mL which is simply the price divided by the product of the OD of the solution and the volume. For example, if a 100 mL volume of gold nanoparticles at a 10 OD concentration costs $600, the cost per OD-mL is $600/(100*10) = $0.60. Unlike a number of other vendors, our covalent coupling solution is extremely cost effective with the final cost being only slightly higher than that of products used for physisorption. When you factor in the advantages of faster development time, increased stability in a wide variety of sample media, lower antibody usage per particle and better control over the antibody/particle ratio, in many cases there is a net cost reduction as well as a performance increase by utilizing covalent binding.

Feel free to contact one of our sales staff to provide quotes or for more help with covalent binding.

CONCLUSION

Our BioReady carboxyl gold is a simple and cost-effective method of creating sensitive, robust and reliable gold conjugates. We have a number of customers who were unable to make a stable conjugate with passive adsorption but were successful with covalent binding. For competitive lateral flow assays where the number of antibodies on the surface of the particle is critical for adjusting the dynamic range of the assay, covalent binding increases the control over the particle/antibody ratio. Since covalent binding is more efficient than passive adsorption, the reduction in the amount of antibody needed to build a test can result in a net decrease in assay cost when using expensive antibodies. In our hands, the biggest advantage of covalent binding is the ability to rapidly sweep antibody pairs as it is no longer necessary to sweep salt and pH for each nanoparticle conjugate – instead standard EDC/NHS binding conditions typically work the first time. If you haven't experimented with covalent binding yet, please contact us for help with particle selection and binding chemistry protocols and support.

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