12 nm NANOGOLD®

Nanoprobes
95 Horseblock Road, Unit 1, Yaphank NY 11980-9710
Tel: (877) 447-6266 (Toll-Free in US) or (631) 205-9490. Fax: (631) 205-9493
Tech Support: (631) 205-9492 tech@nanoprobes.com
www.nanoprobes.com

PRODUCT INFORMATION

12 nm MALEIMIDO NANOGOLD® LABELING REAGENT

Product Name:	**12 nm Maleimido Nanogold®**
Quantity:	**0.1 nmol per vial**
Catalog Number:	**2420A-5X0.1NMOL**
	2420S-0.1NMOL
Appearance:	**Red solid**
Revision:	**1.0 (June 2025)**

Congratulations on your acquisition of a revolutionary new gold labeling reagent: **12 nm Maleimido Nanogold®**. This reagent may be used to label antibodies, proteins, peptides, modified nucleic acids, or any other biomolecule containing an accessible aliphatic thiol (sulfhydryl) group, with the 12 nm Nanogold® gold nanoparticle. Nanogold® is conjugated through discrete, covalent chemical reactions.[1] Unlike conventional colloidal gold, which is non-specifically adsorbed to antibodies and proteins, Nanogold® is selectively conjugated at specific sites. Nanogold® does not require additional macromolecules such as BSA for stabilization, so probes are smaller and the gold label can get closer to the site of interest. Conjugates prepared with this reagent have several advantages over conventional colloidal gold conjugates (see below).

Contents

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals. Non-radioactive and non-carcinogenic.

Product Information

12 nm Maleimido Nanogold® is a recently developed, highly monodisperse gold nanoparticle label that is stabilized by a layer of coordinated organic ligands. This confers high stability, biocompatibility and water-solubility. These coordinated ligands also incorporate reactive Maleimido groups that react selectively with thiols (sulfhydryls), producing a stable thioether bond that permanently cross-links the Nanogold® to the conjugate biomolecule, as shown in Figure 1:

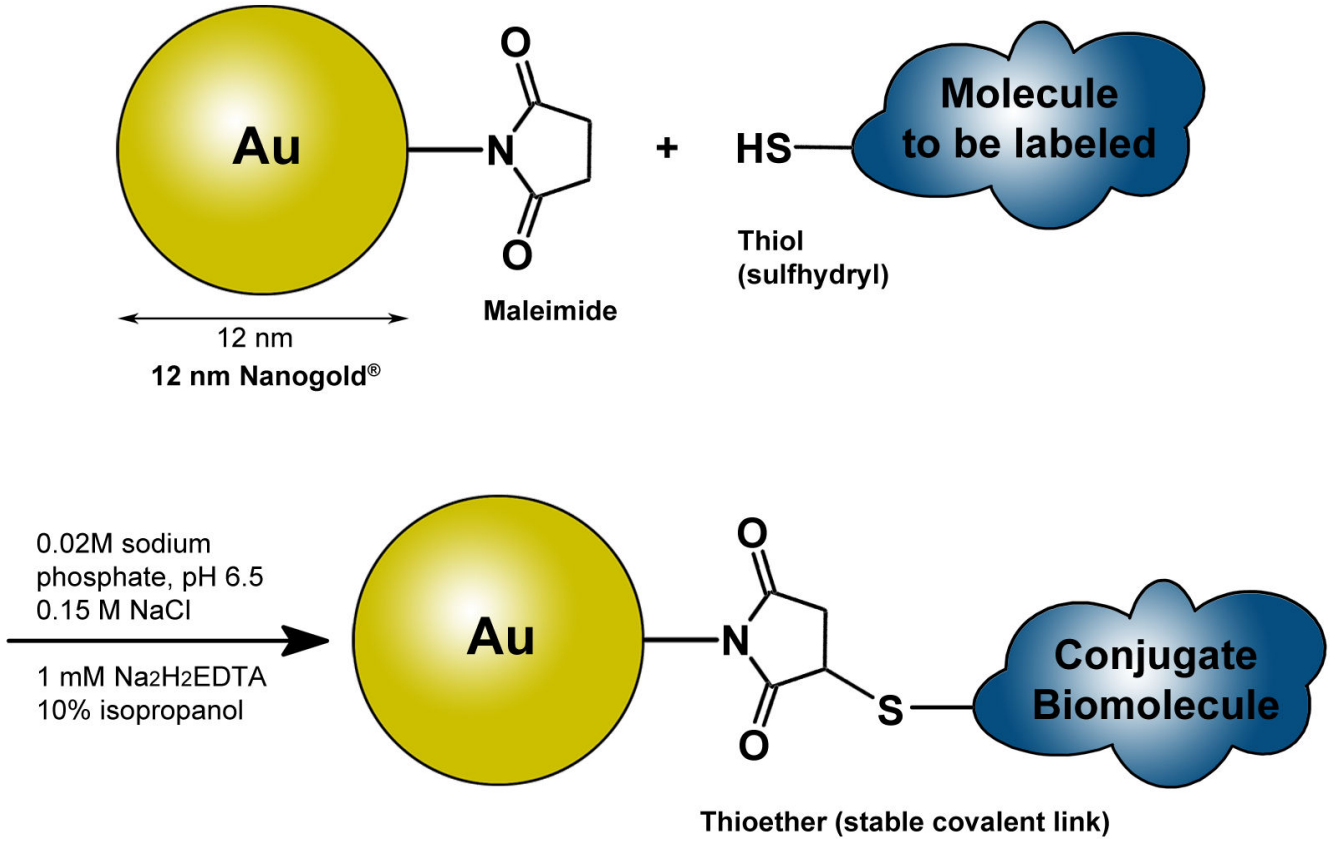

Figure 1: 12 nm Maleimido Nanogold® labeling of a protein via reaction of a thiol with the maleimide group.

12 nm Maleimido Nanogold® may be used to label any molecule with an accessible aliphatic thiol:[2-4]

- Antibodies: label site-specifically at a hinge thiol obtained by selective reduction of a disulfide bridge
- Antibody Fab' fragments: label selectively at the hinge thiol
- Smaller antibody fragments, such as ScFv or other antibody fragments with intra-chain disulfides or cysteine residues.
- Proteins, which may be labeled at the side-chain thiols of cysteine residues.
- Peptides
- Thiol-modified modified nucleic acids (oligonucleotides, DNA, and oligonucleosides, RNA) and peptide nucleic acids (PNA)
- Lipids
- Small molecules such as hormones, toxins, vitamins, growth factors, substrate analogs or signaling molecules
- Surfaces
- Components of self-assembling nanostructured materials

This reagent as supplied has been lyophilized from deionized water and is in the form of a sticky, dark red solid. This should be reconstituted before use in reaction buffer, usually 0.02 M sodium phosphate with 0.15 M sodium chloride (PBS) with 2 mM disodium dihydrogen EDTA (ethylenediaminetetraacetate) at pH 6.5. Nanogold® conjugates can be used in immunoblotting, light microscopy, and electron microscopy to provide clear visibility. They are stable to wide ranges of pH and ionic strength, and are not radioactive or carcinogenic.

12 nm Maleimido Nanogold® should be stored at -20°C.

0.1 nmol of reagent is supplied, sufficient to ensure complete labeling of up to 0.1 nmol of thiol sites; this corresponds to about 0.015 mg of IgG (molecular weight 150 kDa), or 0.01 mg of a protein with a molecular weight of 100,000 with one labeled thiol site.

Nanogold®-labeled biomolecules prepared with 12 nm Maleimido Nanogold® can be enlarged for electron microscopy, or visualized by light microscopy, as well as on gels and blots, using silver or gold enhancement. Nanogold® will nucleate silver or gold metal deposition, resulting in a dense particle up to 80 nm in size or larger depending on development time, and producing a black, brown or purple stain for optical visualization.[5]

Nanoprobes offers a number of silver enhancement and gold enhancement reagents. Detailed instructions may be found with each:

Silver enhancement kits:

2012-45 mL HQ Silver™
Best for EM: Uniform enhancement, low background and excellent ultrastructural preservation.

2013-250 mL LI Silver™
Use to stain Nanogold® labeled proteins or nucleic acids for light microscopic observation, in gels and on blots.

For more information, visit our website:
https://www.nanoprobes.com/products/Silver-Enhancers.html

GoldEnhance™ gold enhancement kits:

2112-28 mL GoldEnhance™ LM
Brown colored stains. High sensitivity and low background.

2113-8 mL GoldEnhance™ EM
High sensitivity and rapid enhancement.

2114-8 mL GoldEnhance™ EM Plus
Uniform enhancement and high sensitivity.

2115-48 mL GoldEnhance™ Blots
Purple colored stain. High sensitivity and rapid enhancement for direct optical and visual detection.

For more information, visit our website:
https://www.nanoprobes.com/products/GoldEnhance.html

Thiol Caution

12 nm Nanogold® particles may be degraded upon prolonged exposure to thiols such as ß-mercaptoethanol or dithiothreitol. If these are required, we recommend that concentrations be kept below 10 mM and exposure times limited to one hour or less. Elevated temperatures may increase the risk of thiol degradation. If possible, monitor reaction mixtures or preparations containing thiols by UV/visible spectroscopy for any change in color, spectral profile, or reduction in absorption at the plasmon resonance peak (close to 520 nm), and remove thiols if any changes are observed.

Thiol compounds used for the reduction of proteins (or other biomolecules) must be separated from reduced proteins by gel filtration liquid chromatography before Nanogold® conjugation. Best results are usually obtained using a desalting gel with an appropriate molecular weight cut-off (MWCO) such as one of the Sephadex gels (Cytiva) or 7K MWCO Zeba spin desalting columns, plates or

cartridges (Thermo Fisher Scientific). Dialysis is not recommended as it frequently does not afford complete thiol removal in this application. **Do not store 12 nm Nanogold® or its conjugates in the presence of thiols**.

Other Cautions

12 nm Nanogold® is stable at temperatures up to 100°C at pH values from 5 to 10 and ionic strengths under 0.3 M, which includes many commonly used or standard biological buffers. However, under demanding conditions, including pH values lower than 4 or ionic strengths above 0.3 M, Nanogold® reagents, conjugates or labeled specimens may be less stable above 50°C, and best results are obtained at room temperature or 4°C. If your experiment requires such conditions, avoid 37°C incubations, and use low temperature embedding media (e.g., Lowicryl) for pre-embedding immunolabeling.[6]

Introduction to Labeling with 12 nm Nanogold®

General considerations

There are important differences between our 12 nm Nanogold® reagents and our smaller, 1.4 nm Nanogold® reagents, which can affect conjugation. Before starting a labeling reaction, you should consider the following:

(a) What is your desired degree of labeling? What ratio of 12 nm Nanogold® : conjugate biomolecule is preferred or acceptable?
(b) Do you require the complete removal of (i) unconjugated 12 nm Nanogold®, and / or (ii) unlabeled conjugate biomolecule?
(c) How will you separate the 12 nm Nanogold® conjugate from unconjugated 12 nm Nanogold® and unlabeled conjugate biomolecule?

Two differences between 12 nm Nanogold® and our smaller 1.4 nm Nanogold® reagents may necessitate changes in your conjugation procedure:

(1) 12 nm Nanogold® reagents are large molecules.

In addition to the 12 nm diameter gold core, 12 nm Nanogold® reagents contain a coating of organic molecules, which forms a 'shell' about 6 nm thick. Their overall diameter, including this layer, is about 24 nm. This is larger than most proteins and biomolecules that are commonly used as probes. For example, IgG, which has a molecular weight (MW) of 150,000 Da, is about 12 nm in length, and each component fragment (Fab, Fab' or Fc) is about 6 nm in length. Based on an average protein density of 1.35 g/cm^3, a globular protein with the same dimensions as 12 nm Nanogold®, a 24 nm diameter sphere, would have a molecular weight of 5.8 x 10^6 Da (5,800 kDa). For comparison, thyroglobulin, which is used as a high molecular weight standard for chromatographic separations, has a molecular weight of 669,000 Da, and occupies a volume about one-eighth that of 12 nm Nanogold®. Since proteins usually are not spherically symmetrical, it is reasonable to expect a protein or protein complex of about 5,000 kDa to have similar size separation behavior to 12 nm Nanogold®.

This is an important consideration in choosing a conjugation strategy for 12 nm Nanogold® conjugates. If you plan to separate conjugates by a size-dependent method such as centrifuge filtration or gel filtration, use an excess of the smaller of the two reagents, because the size difference between it and the conjugate will be greater and it will be easier to remove. The larger reagent is the limiting reagent: since it reacts completely, separation of unreacted larger reagent is not an issue.

(2) 12 nm Nanogold® reagents are multi-functional

Unlike the smaller 1.4 nm monofunctional Nanogold® reagents, each 12 nm gold nanoparticle may contain as many as 50 or more reactive groups. If you are using a 12 nm Nanogold® reagent to label a molecule containing more than one reactive group, then not only can the 12 nm Nanogold® react with a second biomolecule, but if there is a second reactive group on the labeled biomolecule, it may then react with a second 12 nm Nanogold® reagent. This can produce extended oligomers, forming intractable aggregates or precipitates. It is therefore important to use a conjugation strategy that minimizes this risk.

Labeling Strategy

(1) Determine desired degree of labeling and reaction stoichiometry (ratio of 12 nm Nanogold® reagent : molecule to be labeled)

For efficient Nanogold® labeling, use an excess of the reagent that is more easily separated. The reagent that is more difficult to separate is the "limiting" reagent: it reacts completely, and therefore no unreacted material must be separated. Most separation methods, including the simplest and best optimized, are based on size. A greater size difference between the conjugate and unreacted excess reagent usually means higher resolution, easier and more complete separation.

However, your conjugation strategy should also maximize your yield of monomeric conjugates and avoid forming excessively cross-linked oligomers or aggregates:

(a) Conjugate 12 nm Nanogold® to a unique functional group. For example, if the molecule you wish to label has several lysine residues (amines) but only one cysteine (thiol), use 12 nm Maleimido Nanogold® to label at the unique cysteine, rather than using 12 nm *Sulfo*-NHS-Nanogold® to label at an amine.

(b) Take advantage of immobilization. If you can immobilize the molecule to be labeled before conjugation, you will prevent the 12 nm Nanogold® from cross-linking multiple conjugate biomolecules together. If you are using a 12 nm Nanogold® reagent to label a probe that binds a target on a non-reactive surface, allow the probe to bind to this surface, then apply the 12 nm Nanogold®. This approach also allows easy removal of excess 12 nm Nanogold® by washing.

(c) Use a reaction stoichiometry that favors your desired product and separation method:

- If you are labeling a smaller molecule with a single reactive group and require 1 : 1 conjugates (one Nanogold® per conjugate), use a small excess (2-fold) of 12 nm Nanogold®, and use a non-size-dependent separation method, such as ammonium sulfate precipitation or affinity, ion exchange, reverse-phase or hydrophobic interaction chromatography.

- If you are labeling a larger molecule that has a single reactive group, use a small excess (2-fold) of 12 nm Nanogold®. Using excess 12 nm Nanogold® will favor reaction of biomolecules with unconjugated Nanogold® particles, reducing the fraction of reactions between a conjugate biomolecule and an already conjugated 12 nm Nanogold®, while making the conjugate biomolecule the limiting reagent and ensuring that it is completely labeled.

- If you cannot use a unique functional group on the molecule you are labeling, adjust the reaction stochiometry to minimize excessive cross-linking and favor the formation of the desired product. Make sure that you can separate conjugates in a form and purity that meet the needs of your experiment: if either unconjugated 12 nm Nanogold® or unlabeled probe would interfere with your experiment, choose a reaction method that lets you separate them once reaction is complete.

  (i) If you are labeling a smaller molecule that has more than one reactive group and a conjugate with more than one conjugate molecule attached to each 12 nm Nanogold® works for your experiment, use a large excess of the smaller molecule to be labeled (10-fold to 50-fold: use the smaller excess for molecules closer in size to 12 nm Nanogold®, and a larger excess for much smaller molecules).

  (ii) If you are labeling a smaller molecule with more than one reactive group and require 1 : 1 labeling (one 5 nm Nanogold® per conjugate), then use a small excess of the 12 nm Nanogold® (1.5 : 1 or 2 : 1). After it has reacted, the larger bound Nanogold® should hinder approach and reaction with a second Nanogold® sufficiently to favor the formation of monomeric 1 : 1 product. Use a non-size-dependent separation method, such as ammonium sulfate precipitation or affinity, ion exchange, reverse-phase or hydrophobic interaction chromatography, that can separate unconjugated 12 nm Nanogold®. Any larger aggregates may be removed by centrifugation at low g to pellet them, or by filtration.

  (iii) If you are labeling a larger molecule or complex with more than one reactive group and a conjugate with more than one 12 nm Nanogold® per conjugate works for your experiment, use an excess of 12 nm Nanogold® (three-fold to five-fold: use a smaller excess for molecules closer in size to the Nanogold®, and a larger excess for much larger molecules).

  (iv) If you are labeling a larger molecule with more than one reactive group and require 1 : 1 labeling (one 12 nm Nanogold® per conjugate), using close to a 1 : 1 ratio of 12 nm Nanogold® : molecule to be labeled or a small excess of 12 nm Nanogold® (1.5 : 1 or 2 : 1) is likely to give the best yield of desired product. Use a non-size-dependent separation method, such as ammonium sulfate precipitation or affinity, ion exchange, reverse-phase or hydrophobic interaction chromatography. Remove any larger aggregates by centrifugation at low g to pellet them, or by filtration.

(2) Choose a compatible separation method

12 nm Nanogold® conjugates may be separated from unconjugated 12 nm Nanogold® and unlabeled conjugate biomolecule using these methods:

(a) Centrifuge concentration (membrane filtration): If there is a large size difference between the molecule you plan to label and the 12 nm Nanogold® reagent, you can use a centrifuge filter or concentrator to remove the smaller of the reagents. If you are labeling a molecule with a molecular weight of less than 1,000 kDa, a 1,000 kDa molecular weight cut-off (MWCO) filter will retain the conjugate and any unconjugated 12 nm Nanogold®, and filter out unlabeled biomolecule. For pure product, use an excess of the molecule to be labeled to ensure complete reaction of the 12 nm Nanogold®.

(b) Gel filtration (size exclusion) liquid chromatography: If the size difference between the molecule you plan to label and the 12 nm Nanogold® reagent does not allow effective centrifuge filtration, or if you need to characterize the species formed by the reaction, gel filtration (size exclusion) liquid chromatography may effectively isolate conjugates while providing information on the sizes of the different reaction products, although the choice of gels with appropriate molecular weight separation ranges may be limited.

If you plan to use gel filtration, use an excess of the smaller of the two reagents; this will ensure that the larger molecule, or limiting reagent, reacts completely. The chromatographic resolution between the conjugate and unreacted excess smaller reagent will be higher than that between conjugate and unreacted larger reagent.

(i) If you are labeling a smaller molecule (molecular weight 1,000 kDa or less), use an excess (3-fold) of this molecule; this will ensure complete reaction of the 12 nm Nanogold®, and unreacted smaller molecule will be more easily separated.

(ii) If you are labeling a larger molecule or complex (molecular weight 10,000 kDa or more), use an excess (2-fold to 3-fold) of 12 nm Nanogold® reagent; the conjugate molecule will react completely, and unconjugated 12 nm Nanogold® reagent will be more completely separated.

(iii) If the two reagents are similar in size, best results are usually obtained using a small excess (1.5 to 2-fold) of 12 nm Nanogold® reagent.

In general, we find that agarose gels are more compatible with Nanogold® conjugates than acrylate gels. Use a gel where the expected size of the conjugate is near the upper end of the separation range, and the smaller of the two reagents is towards the lower end. Superose-6 (Cytiva), which has a MW fractionation range of 5×10^4 – 5×10^6 Da (exclusion limit 4×10^7 Da) separates 12 nm Nanogold® conjugates, as both conjugates and unconjugated smaller molecules are eluted within the separation range and both may be resolved from any larger oligomers or aggregates. Other gels with appropriate separation characteristics include the larger molecular weight range Bio-Gel columns from Bio-Rad: Bio-Gel A-15m gel (MW separation range 40,000–15,000,000 Da) and Bio-Gel A-50m gel (MW separation range 100,000–50,000,000 Da). Appropriate HPLC columns include TSKgel SuperAW6000 (Tosoh) and Biozen 1.8 µm SEC-3 (Phenomenex).

(c) Other chromatographic methods: other liquid chromatography modalities, such as ion exchange (IEC), reverse-phase or hydrophobic interaction chromatography (HIC), may be useful if size-dependent separation is not possible. If you are using one of these methods, test a small portion of (i) unreacted 12 nm Nanogold® reagent and (ii) unlabeled biomolecule separately before reaction so that you know where each elutes and can identify the conjugate.

(d) Ammonium sulfate precipitation: this will precipitate proteins and conjugates, while leaving unconjugated 12 nm Nanogold® in solution: it is effective when using excess 12 nm Nanogold® reagent. Since it is not size-dependent, it is useful for some reactions where centrifuge filtration or gel filtration liquid chromatography do not provide efficient separation, for example preparation of a 1 : 1 conjugate with a smaller molecule using excess 12 nm Nanogold® reagent.

(e) Gel electrophoresis: may be useful for analytical separations. Note that gel shifts may differ from those expected on the basis of molecular weight and may not be reliable indicators of the success or failure of a conjugation reaction. For best results:

(i) Use a non-reducing gel; thiols can degrade Nanogold® or its conjugates.

(ii) Run two lanes in parallel under identical conditions.

(iii) Divide the gel into two sections each containing one of the two lanes. Stain one section using a protein stain such as Coomassie blue. Develop the other using a silver or gold enhancement reagent (do not use a protein silver stain, as this is a different chemistry and compare the two sections. Conjugate bands will be visualized on both gels. Unlabeled proteins will be visualized only by protein stain, and unconjugated 12 nm Nanogold® only by silver or gold enhancement.

Labeling IgG Molecules with 12 nm Maleimido Nanogold®

IgG molecules contain disulfide bonds that connect the chains in the hinge region. These are selectively reduced with a mild reducing agent, then reacted with 12 nm Maleimido Nanogold® in buffer solution, either for 2 h at room temperature or overnight at 4°C. The coupling reaction should be performed at pH 6.5: at pH values greater than 7, the maleimido group becomes reactive towards primary amines as well as thiols, and may give non-specific labeling. Hydrolysis of the maleimide group also happens faster at higher pH.

12 nm Maleimido Nanogold® is larger than IgG and more than one IgG molecule may be conjugated to each 12 nm Maleimido Nanogold® particle. If you prefer a 1 : 1 IgG : Nanogold® ratio, we recommend using a small (1.5-fold) stoichiometric excess of IgG over 12 nm Maleimido Nanogold®. If you prefer several IgG molecules per Nanogold®, use a larger excess (5-fold to 10-fold). This will ensure complete labeling, and facilitate removal of unconjugated IgG. The 12 nm Nanogold® conjugate may be separated from unlabeled IgG either by gel filtration liquid chromatography using a fine gel such as Cytiva Superose 6, or by ammonium sulfate precipitation.

IgG reduction:

Two methods are available for selectively reducing hinge disulfides in IgG to generate thiols for selective labeling:

(a) Thiol-based reducing agents, such as dithiothreitol (DTT) or mercaptoethylamine hydrochloride (MEA)

If you use a thiol-based reducing agent, the reduced antibody must be separated completely from the reducing agent before reaction; this may be achieved by gel filtration chromatography, using a gel such as Matrex GH25 (Millipore), which has an exclusion cutoff at molecular weight 3,000.

1. Dissolve the required amount of IgG antibody in 0.2 mL of 0.1 M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA. For 1 : 1 IgG : Nanogold®, use 0.015 mg IgG (0.15 nmol); for a higher IgG : Nanogold® ratio, use 0.075 – 0.15 mg (0.5 – 1 nmol). If the IgG is already in solution, exchange into the phosphate/EDTA buffer using membrane centrifugation (e.g. Centricon-50 system, Millipore) or a desalting column. Add 0.2 mL of the IgG solution to a vial containing 3 mg of mercaptoethylamine hydrochloride (MEA) in 0.3 mL of 0.1 M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA (Final concentration 0.05M). Incubate at 37°C for 90 minutes.
2. Isolate reduced antibody by gel filtration chromatography. Use a desalting gel, such as Matrex GH25 (Millipore), which has an exclusion cut-off at molecular weight 3,000. Dialysis does NOT provide acceptable purification in this application. Elute with 0.02 M sodium phosphate at pH 6.5, with 150 mM sodium chloride and 1 mM EDTA. The reduced antibody will be eluted in the void volume as the first sharp peak in the trace. Combine the fractions containing reduced antibody; the total amount of antibody should be calculated from the optical density (usually for IgG, $E_{1\%}$ at 280 nm = 14.5; concentration in mg/mL = $OD_{280\ nm}$ X 10/$E_{1\%}$ = $OD_{280\ nm}$ X 0.69).

(b) Reduction with tris(2-carboxyethyl)phosphine (TCEP)[7]

If you cannot use gel filtration liquid chromatography, tris(2-carboxyethyl)phosphine (TCEP) is an alternative reducing agent that does not react with maleimides, and does not need to be removed before adding Maleimido Nanogold®.[7] Once reduction is complete, proceed to the labeling step. See our website for more details (https://www.nanoprobes.com/newsletters/Vol11_iss01_disulfides.html).

1. Dissolve the required amount of IgG antibody in 0.25 mL of degassed 0.02 M sodium phosphate at pH 7.4, with 150 mM sodium chloride (PBS).
2. Dissolve 1.5 mg of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in 0.25 mL of degassed 0.02 M sodium phosphate at pH 7.4, with 150 mM sodium chloride (PBS) (this will make a 20 mM solution of TCEP). Add the IgG antibody solution, mix gently and incubate for 4 h at 4°C. Adjust the pH to 6.5 or exchange into 0.02 M sodium phosphate at pH 6.5, with 150 mM sodium chloride and 1 mM EDTA for reaction with 12 nm Maleimido Nanogold®.

12 nm Maleimido Nanogold® labeling:

1. Dissolve the 12 nm Maleimido Nanogold® (0.1 nmol) in 0.2 mL of 0.02 M sodium phosphate at pH 6.5, with 150 mM sodium chloride and 1 mM EDTA. If the reagent is slow to dissolve, vortex the solution. Once 12 nm Maleimido Nanogold® is reconstituted with buffer, it should be used immediately. The maleimide group is hydrolyzed in aqueous solution.
2. Add the reconstituted 12 nm Maleimido Nanogold® solution to the reduced antibody immediately. Incubate for 2 hours at room temperature. For an overnight reaction at 4°C, reduce the reaction volume to give an IgG concentration of 6.5

nmol/mL using a membrane centrifugation device with a 30 kDa molecular weight cut-off (e.g. Centricon-30 system, Millipore).

3. Separate the unbound gold particles from the antibody conjugate:

   Ammonium sulfate precipitation: After two hours reaction at room temperature, add saturated ammonium sulfate to 38% of total volume (a 2 mL reaction volume will require 1.226 mL of saturated ammonium sulfate solution). Agitate gently, then centrifuge for 10 minutes at 5000 X g. Remove the supernatant and resuspend the residue in 2 mL PBS.

   Gel filtration (size exclusion) liquid chromatography: use a gel with an appropriate size fractionation range that will efficiently separate 12 nm Nanogold®-IgG from smaller molecules. Choose a separation matrix with a separation range such that 12 nm Nanogold®-IgG elutes towards the upper end of the range. Appropriate gels include Superose 6 (Cytiva), and Bio-Gel A-1.5m (Bio-Rad). Appropriate HPLC columns include TSKgel® SuperAW5000 (Tosoh) and Biozen 1.8 µm SEC-3 (Phenomenex).

   Concentrate the reaction mixture to a suitably small volume for injection using membrane centrifugation (e.g. Amicon Ultra-2, 100,000 MWCO). Elute with 0.02 M sodium phosphate at pH 7.4 with 150 mM sodium chloride; the first, light red peak or shoulder is the conjugate, while the second colorless band is excess unlabeled IgG. For even higher purity, repeat this process one more time.

To calculate the labeling efficiency (ratio of 12 nm Nanogold® : IgG in the conjugate product), see below, in the section titled "Characterization of 12 nm Nanogold® Conjugates."

Nanogold® conjugates should be stored at 4°C in 0.02 M sodium phosphate buffer at pH 7.4 with 150 mM sodium chloride; if the protein concentration is less than 1 mg/mL, add 0.1% bovine serum albumin to stabilize the proteins, and 0.05 % sodium azide to prevent bacterial contamination.

Labeling Fab' Fragments with 12 nm Maleimido Nanogold®

If F(ab')$_2$ fragments are available, they should be used for this method. If F(ab')$_2$ fragments are not available, they may be prepared from IgG molecules by pepsin[8] or ficin[9] digestion. It should be noted that IgG molecules from different host animals vary slightly in structure and therefore will differ in their ease of digestion. The procedure given below has been found to be effective for the preparation of F(ab')$_2$ fragments from IgG1 and IgG2a subclasses. Pepsin becomes more active as the pH is lowered; at pH 7 it is inactivated. Note that some monoclonal antibody subclasses may be unsuitable for this process (for example, IgG3); a comprehensive review has been published on the use of pepsin digestion to prepare F(ab')$_2$ fragments from mouse monoclonal IgG molecules that details which classes may be digested.[8]

Because 12 nm Maleimido Nanogold® is significantly larger than Fab' fragments, unless 1 : 1 labeling is a priority, we recommend using a stoichiometric excess (5-fold to 20-fold) of Fab' fragments over 12 nm Maleimido Nanogold®. This will ensure complete labeling and facilitate removal of unconjugated Fab' fragments. 12 nm Nanogold® - Fab' conjugates may be separated from unlabeled Fab' fragments by centrifuge filtration using a 100 kDa MWCO filter, by gel filtration liquid chromatography using a fine gel such as Cytiva Superose 6, or by ammonium sulfate precipitation.

F(ab')$_2$ preparation from IgG:

1. Dissolve IgG in 0.1 M sodium citrate buffer at pH 4.5; add a solution of pepsin in 0.5 mL of the same buffer. Use an amount of pepsin equal to 2% of the mass of IgG. Incubate at 37°C for 20 h using a water bath or incubator.
2. Isolate the F(ab')$_2$ fragments by gel filtration chromatography, using a column such as Pharmacia Superose 6 or 12, TSK 3000, or Toyozoda MacMod GF-250 (which have wide molecular weight fractionation ranges) or a superfine exclusion gel such as Matrex GCL300 (Millipore; excludes compounds with molecular weights above 60,000). Dialysis does NOT provide acceptable purification in this application.

   Elute with 0.1 M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA. If a GCL300 column is used, The F(ab')$_2$ fragments will elute in the void volume as the first band. Combine the fractions containing F(ab')$_2$ fragments and calculate the amount using the optical density. Concentrate to 5% or less of the column volume (0.5 mL or less for a 10 mL column) or less using membrane centrifugation.

F(ab')$_2$ reduction:

Two methods are available for selectively reducing hinge disulfides in F(ab')$_2$ fragments to generate thiols for selective labeling:

(a) Thiol-based reducing agents, such as dithiothreitol (DTT) or mercaptoethylamine hydrochloride (MEA)

If you use a thiol-based reducing agent, the Fab' fragments must be separated completely from the reducing agent before reaction; this may be achieved by gel filtration chromatography, using a gel such as Matrex GH25 (Millipore), which has an exclusion cutoff at molecular weight 3,000.

1. Dilute the required amount of $F(ab')_2$ fragments in 0.2 mL of 0.1 M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA. For 1 : 1 Fab' : Nanogold®, use 0.075 mg $F(ab')_2$ fragments (0.15 nmol of Fab' fragments); for a higher Fab' : Nanogold® ratio, use 0.025 – 0.05 mg (0.5 – 1 nmol of Fab' fragments). If the $F(ab')_2$ is already in solution, exchange into the phosphate/EDTA buffer using membrane centrifugation (e.g. Centricon-50 system, Millipore) or a desalting column. Add 0.2 mL of the $F(ab')_2$ solution to a vial containing 3 mg of mercaptoethylamine hydrochloride (MEA) in 0.3 mL of 0.1 M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA (Final concentration 0.05M). Incubate at 37°C for 90 minutes.
2. Isolate reduced antibody by gel filtration chromatography. Use a desalting gel, such as Matrex GH25 (Millipore), which has an exclusion cut-off at molecular weight 3,000. Dialysis does NOT provide acceptable purification in this application. Elute with 0.02 M sodium phosphate at pH 6.5, with 150 mM sodium chloride and 1 mM EDTA. The reduced antibody will be eluted in the void volume as the first sharp peak in the trace. Combine the fractions containing reduced antibody; the total amount of antibody should be calculated from the optical density (usually for Fab', $E_{1\%}$ at 280 nm = 15.3; concentration in mg/mL = $OD_{280\ nm}$ X $10/E_{1\%}$ = $OD_{280\ nm}$ X 0.65).

(b) Reduction with tris(2-carboxyethyl)phosphine (TCEP)[7]

If you cannot use gel filtration liquid chromatography, tris(2-carboxyethyl)phosphine (TCEP) is an alternative reducing agent that does not react with maleimides, and does not need to be removed before adding Maleimido Nanogold®.[7] Once reduction is complete, proceed to the labeling step. See our website for more details (https://www.nanoprobes.com/newsletters/Vol11_iss01_disulfides.html).

1. Dilute the required amount of $F(ab')_2$ fragments in 0.25 mL of degassed 0.02 M sodium phosphate at pH 7.4, with 150 mM sodium chloride (PBS).
2. Dissolve 1.5 mg of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in 0.25 mL of degassed 0.02 M sodium phosphate at pH 7.4, with 150 mM sodium chloride (PBS) (this will make a 20 mM solution of TCEP). Add the $F(ab')_2$ fragments in solution, mix gently and incubate for 4 h at 4°C. Adjust the pH to 6.5 or exchange into 0.02 M sodium phosphate at pH 6.5, with 150 mM sodium chloride and 1 mM EDTA for reaction with 12 nm Maleimido Nanogold®.

12 nm Maleimido Nanogold® labeling:

5. Dissolve the 12 nm Maleimido Nanogold® (0.1 nmol) in 0.2 mL of 0.02 M sodium phosphate at pH 6.5, with 150 mM sodium chloride and 1 mM EDTA. If the reagent is slow to dissolve, vortex the solution. Once 12 nm Maleimido Nanogold® is reconstituted with water it should be used immediately. The maleimide group is hydrolyzed in aqueous solution.
6. Add the reconstituted 12 nm Maleimido Nanogold® solution to the Fab' solution immediately. Incubate for 2 hours at room temperature. For an overnight reaction at 4°C, reduce the reaction volume to give a Fab' concentration of 20 nmol/mL using a membrane centrifugation device with a 10 kDa molecular weight cut-off (e.g. Centricon-10 system, Millipore).
7. Separate any excess unconjugated Fab' fragments from the 12 nm Nanogold® conjugate:

   Centrifuge concentration (membrane filtration): After two hours reaction at room temperature, concentrate the solution to minimum volume using a 100,000 molecular weight cut-off (MWCO) centrifuge concentrator. Repeat twice for higher purity. Resuspend the concentrate in 0.5 mL 0.02 M sodium phosphate buffer at pH 7.4 with 150 mM sodium chloride (PBS).

   Ammonium sulfate precipitation: After two hours reaction at room temperature, add saturated ammonium sulfate to 38% of total volume (a 2 mL reaction volume will require 1.226 mL of saturated ammonium sulfate solution). Agitate gently, then centrifuge for 10 minutes at 5000 X g. Remove the supernatant and resuspend the residue in 0.5 mL 0.02 M sodium phosphate buffer at pH 7.4 with 150 mM sodium chloride (PBS).

   Gel filtration (size exclusion) liquid chromatography: use a gel with an appropriate size fractionation range that will efficiently separate 12 nm Nanogold®-Fab' from smaller molecules, such that 12 nm Nanogold®-Fab' is excluded or elutes towards the upper end of the range. Appropriate gels include Superose 6 (Cytiva) and Bio-Gel A-1.5m (Bio-Rad). Appropriate HPLC columns include TSKgel® SuperAW5000 (Tosoh) and Biozen 1.8 µm SEC-3 (Phenomenex).

   Concentrate the reaction mixture to a suitably small volume for injection using membrane centrifugation (e.g. Amicon Ultra-2, 50,000 or 100,000 MWCO). Elute with 0.02 M sodium phosphate buffer at pH 7.4 with 150 mM sodium chloride (PBS);

the first, light red peak or shoulder is the conjugate, while the second colorless band is excess unlabeled Fab'. For even higher purity, repeat this process one more time.

To calculate the labeling efficiency (ratio of 12 nm Nanogold® : Fab' in the conjugate product), see "Characterization of 12 nm Nanogold® Conjugates," below.

Nanogold® conjugates should be stored at 4°C in 0.02 M sodium phosphate buffer at pH7.4 with 150 mM sodium chloride (PBS); if the protein concentration is less than 1 mg/mL, add 0.1% bovine serum albumin to stabilize the proteins, and 0.05% sodium azide to prevent bacterial contamination.

Labeling Proteins or Other Molecules with 12 nm Maleimido Nanogold®

12 nm Maleimido Nanogold® may be used to label any protein with an accessible sulfhydryl group, such as a cysteine residue, in the same manner as described above for antibodies.

Amount of protein to use

If you are labeling a protein or other biomolecule and plan to separate the 12 nm Nanogold®-labeled product by centrifuge concentration (membrane filtration), gel filtration liquid chromatography or ammonium sulfate precipitation, prepare the following volumes of protein solution:

Proteins with a unique reactive thiol – centrifuge concentration (membrane filtration) or gel filtration separation:

(a) If the protein to be labeled has just one reactive thiol and is larger than about 5,000 kDa (5,000,000 MW), conjugates should be separated by ammonium sulfate precipitation or a non-size-dependent chromatographic method such as affinity, reverse-phase or hydrophobic interaction chromatography. Gel filtration liquid chromatography is usually not an option because there are no gels with an appropriate MW separation range. Use a small excess (2-fold) of 12 nm Maleimido Nanogold® (i.e. 0.05 nmol of protein).

(b) If the protein to be labeled has just one unique thiol and is smaller than about 150 kDa (150,000 MW), and you plan to use gel filtration to separate conjugates, use an excess of the conjugate biomolecule, as this will ensure complete reaction of the 12 nm Maleimido Nanogold® (the limiting reagent) and easy removal of excess unreacted conjugate biomolecule. For biomolecules closer in size to 12 nm Maleimido Nanogold®, or for 1 : 1 labeling, use a small excess (2-fold, or 0.2 nmol of protein); for smaller biomolecules (less than 100 kDa) or conjugation of multiple biomolecules to each Nanogold®, use a larger excess (5-fold to 10-fold, or 0.5 – 1 nmol of protein).

Proteins with a unique reactive thiol – ammonium sulfate precipitation:

(c) Use a small excess (2-fold) of 12 nm Maleimido Nanogold® to give a 1 : 1 ratio of 12 nm Nanogold® : protein. This should ensure the highest yield of Nanogold® conjugate while minimizing oligomer formation. To conjugate multiple proteins to each Nanogold®, use an excess of protein about two times that of the desired protein : 12 nm Nanogold® ratio (e.g. use an 8 : 1 protein : 12 nm Maleimido Nanogold® ratio for four proteins per 12 nm Maleimido Nanogold®.

Proteins with more than one reactive thiol:

(d) If the protein or structure to be labeled has more than one accessible thiol and is similar in size or larger than about 12 nm Maleimido Nanogold® (i.e. 5,000 kDa or larger) use an excess of 12 nm Maleimido Nanogold®. For proteins similar in size, use a small (2-fold) excess (i.e. 0.05 nmol of protein), and for much larger proteins or complexes use a 5-fold excess (i.e. 0.02 nmol of protein). This will help avoid oligomerization and aggregation through multiple cross-linking.

(e) If the protein to be labeled has more than one accessible thiol and is smaller than about 1,000 kDa (1,000,000 MW): to minimize aggregation, use an excess of the conjugate biomolecule to favor reaction with a different molecule over reaction with a second 12 nm Maleimido Nanogold® through a second thiol. For biomolecules close in size to 12 nm Maleimido Nanogold®, use a small excess (2-fold, i.e. 0.2 nmol of protein); for smaller biomolecules (less than 50,000 MW), use a larger excess (5-fold, i.e. 0.5 nmol of protein).

If you are labeling an oligonucleotide and plan to separate the 12 nm Nanogold®-labeled product by ethanol precipitation, ensure that only one thiol site is available for reaction. For a 1 : 1 conjugate, use a 1 : 1 ratio of 12 nm Maleimido Nanogold® : thiol-modified oligonucleotide (i.e. 0.1 nmol). This should ensure the highest yield of Nanogold® conjugate and the smallest amounts of unreacted starting materials. For multiple oligonucleotide conjugation, use excess thiol-modified oligonucleotide.

Procedure

In some proteins the sulfhydryl functionality is in the form of a disulfide group; this must be reduced with a reducing agent, such as dithiothreitol (DTT), mercaptoethylamine hydrochloride (MEA) or tris(2-carboxyethyl)phosphine hydrochloride (TCEP) before it can be labeled. If you are unsure of the structure of your protein, and have sufficient quantity available, it is recommended that the suitability of the sulfhydryl for labeling be determined first; some sulfhydryl sites may be buried within the protein structure, and therefore inaccessible to the Nanogold® reagent. The suitability of a particular protein for Nanogold® labeling may be determined using ^{14}C iodoacetic acid before labeling is tried; alternatively, a sensitive colorimetric procedure exists for sulfhydryl determination.[10]

1. Dissolve the antibody or protein to be labeled in 0.02 M sodium phosphate at pH 6.5, with 150 mM sodium chloride and 1 mM EDTA, or an alternative buffer, adjusted to a pH between 6.0 amd 7.0 (0.2 mL).
2. Dissolve the 12 nm Maleimido Nanogold® in 0.2 mL 0.02 M sodium phosphate at pH 6.5, with 150 mM sodium chloride and 1 mM EDTA or an alternative buffer, adjusted to a pH between 6.0 amd 7.0. If the reagent is slow to dissolve, vortex or sonicate the solution. Once the 12 nm Maleimido Nanogold® is reconstituted with buffer, it should be used immediately. The maleimido group is hydrolyzed in aqueous solution.

   If solubility is known to be an issue with the molecule you are labeling, solution may be improved by predissolving the 12 nm Maleimido Nanogold® in up to 10% of the final reaction volume (0.04 mL) of ispropanol, then making up to 0.2 mL with buffer.
3. Add the reconstituted 12 nm Maleimido Nanogold® solution to the dissolved protein. Adjust the reaction volume to give a protein concentration of at least 0.2 nmol/mL (for a 150,000 MW protein, this is 0.03 mg/mL) to achieve optimum labeling. A membrane centrifugation unit may be used to reduce the volume of the reaction mixture; a 100,000 nominal molecular weight cut-off (MWCO) filter is recommended (e.g. Amicon Ultra-2, 100,000 MWCO, molecular weight cut-off, from Thermo Fisher or EMD Millipore) to ensure retention of protein and Nanogold® reagent.
4. Incubate for 2 hours at room temperature. Reaction mixture may then be stored overnight (12-18 hours) at 4°C.
5. Separate the unbound gold particles from the protein conjugate:

   Centrifuge concentration (membrane filtration): use when labeling smaller molecules. For labeling 50 kDa or lower molecular weight, use a 100,000 molecular weight cut-off (MWCO) centrifuge concentrator; for molecules 50 – 500 kDa, use a 1,000,000 molecular weight cut-off (MWCO) concentrator. Repeat twice for higher purity. Resuspend the concentrate in 1 mL 0.02 M sodium phosphate buffer at pH 7.4 with 150 mM sodium chloride (PBS).

   Ammonium sulfate precipitation: After two hours reaction at room temperature, add saturated ammonium sulfate to 38% of total volume (a 2 mL reaction volume will require 1.226 mL of saturated ammonium sulfate solution). Agitate gently, then centrifuge for 10 minutes at 5000 X g. Remove the supernatant and resuspend the residue in 2 mL 0.02 M sodium phosphate buffer at pH 7.4 with 150 mM sodium chloride (PBS).

   Gel filtration (size exclusion) liquid chromatography: 12 nm Nanogold® reagents and conjugates are large molecules which are excluded from most gels. Therefore, this method is usually appropriate only for separating excess unlabeled smaller molecules. Use a gel with an appropriate size fractionation range to give efficient separation of conjugate from unlabeled biomolecule, where the unconjugated protein elutes towards the lower end of the range. For example, small molecules (molecular weights less than 10,000) may be separated using gels such as Superdex-75 or Superose-12 (Cytiva). For larger proteins with molecular weights greater than 100,000, appropriate gels include Superose 6 (Cytiva) and Bio-Gel A-1.5m, A-5m, A-15m or A-50m (Bio-Rad). Appropriate HPLC columns include TSKgel® SuperAW5000 (Tosoh) and Biozen 1.8 µm SEC-3 (Phenomenex).

   Concentrate the reaction mixture to a suitably small volume for injection using membrane centrifugation (e.g. Amicon Ultra-2, 50,000 or 100,000 MWCO). Elute with 0.02 M sodium phosphate buffer at pH 7.4 with 150 mM sodium chloride (PBS); the first, red peak or shoulder is the conjugate, while the second, usually colorless band is excess unlabeled proteins. For even higher purity, repeat this process one more time.

   Oligonucleotide conjugates may be separated by ethanol precipitation.

Characterization of 12 nm Nanogold® Conjugates

UV/visible absorption spectroscopy

Identification of 12 nm Nanogold® and conjugates: 12 nm Nanogold® and its conjugates with antibodies, proteins, peptides or oligonucleotides are normally red colored. They can be detected by UV-Visible spectroscopy in the wavelength range 250 - 800 nm. Unlike the spectra of unlabeled antibodies, proteins, peptides or oligonucleotides, which are usually near or at baseline from 300 - 800 nm, 12 nm Nanogold® and its conjugates have significant absorbtion over the range of 300 - 800 nm, with a characteristic broad absorption maximum (λ_{max}) close to 520 nm (the plasmon resonance).

Quantitation of 12 nm Nanogold®: To calculate the amount of 12 nm Nanogold®, measure the absorption at λ_{max} and use the molar extinction coefficient at λ_{max} of 1.7 x 10^8 $M^{-1}cm^{-1}$ to calculate the molar concentration of 12 nm Nanogold®:

[12 nm Nanogold®] = $[A_{\lambda max}]/1.7 \times 10^8$

The molar extinction coefficients of 12 nm Nanogold® at 260 nm and 280 nm are very large relative to those of proteins and oligonucleotides, and therefore the ratio of absorption at these wavelengths to that at λ_{max} does not afford an accurate method for estimating absorption due to proteins or oligonucleotides and hence their concentration, or for calculating degree of labeling.

Negative stain electron microscopy

High-resolution electron microscopy with negative staining may provide a visual confirmation of successful conjugation to large proteins, protein complexes, or other large particles such as viruses. For best results, we recommend our NanoVan™ (vanadium based)[11] or Nano-W™ (tungsten based) stains.

Gel Electrophoresis

Purified 12 nm Nanogold® conjugates or 12 nm Nanogold® conjugate mixtures may also be characterized using SDS gel, native gel or agarose gel. For best results, follow the procedure below:

1. Use a gel with two panels or lanes. Load purified 12 nm Nanogold® conjugate, or Nanogold® conjugate mixture with unlabeled peptide, protein or oligonucleotide and unreacted 12 nm Nanogold® reagent into the left panel of the gel.

2. Duplicate the loading in the same sequence and amounts into the right panel.

3. Run the gel to reach separation. 12 nm Nanogold® has negligible charge, and contributes little to the charges of labeled peptides, proteins or polynucleotides. **Caution**: 12 nm Nanogold® conjugates and reagents should not be heated with ß-mercaptoethanol before loading onto gels as ß-mercaptoethanol can degrade 12 nm Nanogold® particles during incubation.

4. After running the gel to reach separation, cut the gel in the middle to separate the two lanes.

5. Wash one panel with deionized water for 3 x 15 minutes, then incubate this panel with LI silver™ (Nanoprobes Catalog #2013-250 mL) for 10 minutes or with GoldEnhance™ EM (Nanoprobes Catalog #2113-8 mL) according to product instructions. Wash with deionized water for 4 x 5 minutes and continue overnight. The 12 nm Nanogold® conjugate and 12 nm Nanogold® reagent bands will become brown or black in color upon incubation with LI Silver™ or GoldEnhance™ EM.

6. The other panel should be stained either with Coomassie stain (for proteins) or ethidium bromide (for nucleic acids). 12 nm Nanogold® conjugates and unlabeled peptide, protein or nucleic acid will be stained.

12 nm Nanogold® conjugate bands will be stained by both LI Silver™ and Coomassie or nucleic acid stains.

Blotting

Immunodot blots may be used to confirm that 12 nm Nanogold® conjugates bind to their intended target. Suitable procedures are given below in the section titled “Immunoblotting.”

General Considerations for Using 12 nm Nanogold® Reagents

- 12 nm Nanogold® is an extremely uniform 12 nm diameter gold particle (±10%). Including its organic coating, its total diameter is about 24 nm. It is water-soluble, biocompatible and non-ionizing.
- 12 nm Nanogold® is covalently attached to the conjugate peptide, protein, oligonucleotide or other structure after reaction.
- 12 nm Nanogold® conjugates contain no aggregates. This is in sharp contrast to colloidal gold conjugates that usually are prepared by centrifugation to remove the largest aggregates and frequently contain smaller aggregates.
- 12 nm Nanogold® particles do not have affinity to proteins as do colloidal golds. This reduces background and false labeling.
- 12 nm Nanogold® develops better with silver than do most other colloidal golds giving it higher sensitivity. Both silver and gold enhancement can be used to enlarge Nanogold® to desirable sizes for electron and light microscopy, gel and blot detection.

Using Stains with 12 nm Nanogold®

Specimens labeled with 12 nm Nanogold® may be stained with all commonly used counterstains, such as OsO_4, uranyl acetate or lead citrate. For highest contrast and best visualization, we recommend:

1. Use of reduced amounts or concentrations of usual stains.
2. For negative stain EM, use our NanoVan™ (vanadium based)[11] or Nano-W™ (tungsten based) stains.
3. If needed, enhance using our HQ Silver™ or GoldEnhance™ EM or EM Plus products.

Electron Microscopy Immunolabeling with 12 nm Nanogold® Conjugates

If aldehyde-containing reagents have been used for fixation, these must be quenched before immunolabeling. This may be achieved by incubating the specimens for 5 minutes in 50 mM glycine solution in PBS (pH 7.4). Ammonium chloride (50 mM) or sodium borohydride (0.5 - 1 mg/mL) in PBS may be used instead of glycine.

Cells in Suspension

1. Optional fixing of cells: e.g., with glutaraldehyde (0.05 - 1% for 15 minutes) in PBS. Do not use Tris buffer since this contains an amine. After fixation, centrifuge cells (e.g. 1 mL at 10^7 cells/ml) at 300 X g, 5 minutes; discard supernatant; resuspend in 1 mL buffer. Repeat this washing (centrifugation and resuspension) 2 times.
2. Incubate cells with 0.02 M glycine in PBS (5 mins). Centrifuge, then resuspend cells in PBS-BSA buffer (specified below) for 5 minutes.
3. Wash cells using PBS-BSA as described in step 1 (2 X 5 mins). Resuspend in 1 mL Buffer 1.
4. Place 50 - 200 µL of cells into Eppendorf tube. Dilute 12 nm Nanogold® conjugate ~ 50 times in PBS-BSA buffer and add 30 µL to cells; incubate for 30 minutes with occasional shaking (do not create bubbles which will denature proteins).
5. Wash cells in PBS-BSA as described in step 1 (2 X 5 mins).
6. Fix cells and antibodies using a final concentration of 1% glutaraldehyde in PBS for 15 minutes. Then remove fixative by washing with buffer 1 (3 X 5 mins).

PBS-BSA Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4
0.5% BSA
0.1% gelatin (high purity)
Optional, may reduce background:
0.5 M NaCl
0.05% Tween 20

PBS Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4

Negative staining may be used for electron microscopy of small structures or single molecules which are not embedded. Negative stain must be applied after the silver enhancement. For best results, we recommend our NanoVan™ (vanadium-based)[11] or Nano-W (tungsten-based) negative stains.

CAUTION: 12 nm Nanogold® particles may degrade upon exposure to concentrated thiols such as ß-mercaptoethanol or dithiothreitol. If such reagents must be used, concentrations should be kept below 1 mM and exposure restricted to 10 minutes or less.

Thin Sections

Labeling with 12 nm Nanogold® may be performed before or after embedding.[12,13] Labeling before embedding and sectioning (the pre-embedding method) is used for the study of surface antigens, particularly small organisms such as viruses budding from host cells. It gives good preservation of cellular structure, and subsequent staining usually produces high contrast for study of the cellular details. Labeling after embedding and sectioning (the post-embedding method) allows the antibody access to the interior of the cells, and is used to label both exterior and interior features. The procedures for both methods are described below.

Thin sections mounted on grids are floated on drops of solutions on parafilm or in well plates. Hydrophobic resins usually require pre-etching.

CAUTION: Nanogold particles may degrade upon exposure to concentrated thiols such as ß-mercaptoethanol or dithiothreitol. If such reagents must be used, concentrations should be kept below 1 mM and exposure restricted to 10 minutes or less.

PROCEDURE FOR PRE-EMBEDDING METHOD:[12]

1. Float on a drop of water for 5 - 10 minutes.
2. Incubate cells with 1% bovine serum albumin in PBS buffer at pH 7.4 for 5 minutes; this blocks any non-specific protein binding sites and minimizes non-specific antibody binding.
3. Rinse with PBS-BSA (1 min).
4. Incubate with 12 nm Nanogold® conjugate diluted 1/40 - 1/200 in PBS-BSA with 1 % normal serum from the same species as the 12 nm Nanogold® labeled antibody, for 10 minutes to 1 hour at room temperature.
5. Rinse with PBS-BSA (3 X 1 min), then PBS (3 X 1 min).
6. Postfix with 1% glutaraldehyde in PBS (10 mins).
7. Rinse in deionized water (2 X 5 min).
8. Dehydrate and embed according to usual procedure. Use of a low-temperature resin (e.g. Lowicryl) is recommended.
9. Stain (uranyl acetate, lead citrate or other positive staining reagent) as usual before examination.

Silver enhancement may be performed before or after embedding (see below); it should be completed before postfixing or staining with osmium tetroxide, uranyl acetate or similar reagents is carried out.

PROCEDURE FOR POST-EMBEDDING METHOD:[12]

1. Prepare sections on plastic or carbon-coated nickel grid. Float on a drop of water for 5 - 10 minutes.
2. Incubate with 1% solution of bovine serum albumin in PBS buffer at pH 7.4 for 5 minutes to block non-specific protein binding sites.
3. Rinse with PBS-BSA (1 min).
4. Incubate with 12 nm Nanogold® conjugate diluted 1/40 - 1/200 in PBS-BSA with 1% normal serum from the same species as the 12 nm Nanogold® labeled antibody, for 10 minutes to 1 hour at room temperature.
5. Rinse with PBS (3 X 1 min).
6. Postfix with 1% glutaraldehyde in PBS at room temperature (3 mins).
7. Rinse in deionized water for (2 X 5 min).
8. If desired, contrast sections with uranyl acetate and/or lead citrate before examination.

Silver or gold enhancement may also be used to enlarge the 12 nm Nanogold® particles (see below). If used, it should be completed before counterstains such as uranyl acetate or lead citrate are applied.

PBS-BSA Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4
0.5% BSA
0.1% gelatin (high purity)
Optional, may reduce background:
0.5 M NaCl
0.05% Tween 20

PBS Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4

Silver or Gold Enhancement of 12 nm Nanogold® for EM

Silver enhancement

12 nm Nanogold® will nucleate silver deposition resulting in a dense particle 15-40 nm in size or larger, depending on development time. In pre-embedding labeling procedures, silver enhancement may be performed before or after embedding. Silver enhancement must be completed before any staining reagents such as osmium tetroxide, lead citrate or uranyl acetate are applied, since these will nucleate silver deposition in the same manner as gold and produce non-specific staining. With 12 nm Nanogold® reagents, low-temperature resins (e.g. Lowicryl) should be used and the specimens kept at or below room temperature until after silver development has been completed. Silver enhancement is recommended for applications of 12 nm Nanogold® in which these stains are to be used, otherwise the 12 nm Nanogold® particles may be difficult to visualize against the stain.

Best results in the EM may be obtained using HQ Silver™, which is formulated to give slower, more controllable particle growth and uniform particle size distribution.[14] Our LI Silver™ kit is convenient and not light sensitive, and suitable for all applications.

Specimens must be thoroughly rinsed with deionized water before silver enhancement reagents are applied. This is because the buffers used for antibody incubations and washes contain chloride ions and other anions which form insoluble precipitates with silver. These are often light-sensitive and will give non-specific staining. To prepare the developer, mix equal amounts of the enhancer and initiator immediately before use. 12 nm Nanogold® will nucleate silver deposition resulting in a dense particle 15-40 nm in size or larger depending on development time. Use nickel grids (not copper).

The procedure for immunolabeling should be followed up to step 6 as described above. Silver enhancement is then performed as follows:

1. Rinse with deionized water (2 X 5 mins).
2. OPTIONAL (may reduce background): Rinse with 0.02 M sodium citrate buffer, pH 7.0 (3 X 5 mins).
3. Float grid with specimen on freshly mixed developer for 1-8 minutes, or as directed in the instructions for the silver reagent. More or less time can be used to control particle size. A series of different development times should be tried, to find the optimum time for your experiment. With HQ Silver, a development time of 6 mins gives 15-40 nm round particles.
4. Rinse with deionized water (3 X 1 min).
5. Mount and stain as usual.

Fixing with osmium tetroxide may cause some loss of silver; if this is found to be a problem, slightly longer development times may be appropriate.

NOTE: Treatment with osmium tetroxide followed by uranyl acetate staining can lead to much more drastic loss of the silver-enhanced 12 nm Nanogold® particles. This may be prevented by gold toning:[15]

1. After silver enhancement, wash thoroughly with deionized water.
2. 0.05% gold chloride: 10 minutes at 4°C.
3. Wash with deionized water.
4. 0.5% oxalic acid: 2 mins at room temperature.

5. 1% sodium thiosulfate (freshly made) for 1 hour.
6. Wash thoroughly with deionized water and embed according to usual procedure.

Gold Enhancement

The following procedure has been found to give excellent results for pre-embedding immunolabeling using GoldEnhance™ EM Plus, and also works well with GoldEnhance™ EM.[16] Follow the procedure for immunolabeling up to step 4 (Nanogold® conjugate incubation) as described above, then conduct gold enhancement as follows:

1. Wash three times in PBST.
2. Fix 1 hour in 1.2 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, with 5 % sucrose.
3. Wash two times with water.
4. Enhance using GoldEnhance EM Plus (6 minutes).
5. Post-fix in 2 % osmium tetroxide and 2 % potassium ferricyanide in the same buffer.
6. Dehydrate, embed in Epon 812, section and stain with lead citrate before examination in the electron microscope.

Immunolabeling and Silver or Gold Enhancement with 12 nm Nanogold® for Light Microscopy

Light Microscopy with Silver Enhancement

Nanoprobes LI Silver™ kit is simple, convenient and not light sensitive, and suitable for all applications Features labeled with 12 nm Nanogold® will be stained black in the light microscope upon silver enhancement. Different development times should be tried to determine which is best for your experiment. The immunolabeling procedure is similar to that for EM; an example is given below.

Samples must be rinsed with deionized water before silver enhancement. This is because the reagent contains silver ions in solution, which react to form a precipitate with chloride, phosphate and other anions which are common components of buffer solutions.

1. Spin cells onto slides using Cytospin, or use paraffin section.
2. Incubate with 1% w/v bovine serum albumin or in PBS (PBS-BSA) or 1% nonfat dried milk in PBS (PBS-Milk) for 10 minutes to block non-specific protein binding sites.
3. Rinse with PBS-BSA or PBS-Milk (3 X 2 min).
4. Incubate with 12 nm Nanogold® reagent diluted 1/40 - 1/200 in PBS-BSA with 1% normal serum from the same species as the 5 nm Nanogold® reagent, for 1 hour at room temperature.
5. Rinse with PBS (3 X 5 min).
6. Postfix with 1% glutaraldehyde in PBS at room temperature (3 mins).
7. Rinse with deionized water (3 X 1 min).
8. OPTIONAL (may reduce background): Rinse with 0.02 M sodium citrate buffer, pH 7.0 (3 X 5 mins).
9. Develop specimen with freshly mixed developer for 5-20 minutes, or as directed in the instructions for the silver reagent. More or less time can be used to control intensity of signal. A series of different development times may be used, to find the optimum enhancement for your experiment; generally a shorter antibody incubation time will require a longer silver development time.
10. Rinse with deionized water (2 X 5 mins).
11. The specimen may now be stained if desired before examination, with usual reagents.

PBS-BSA Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4
0.5% BSA
0.1% gelatin (high purity)

Optional, may reduce background:

0.5 M NaCl
0.05% Tween 20

PBS Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4

To obtain an especially dark silver signal, the silver enhancement may be repeated with a freshly mixed portion of developer.

Light Microscopy with Gold Enhancement

Nanoprobes GoldEnhance™ LM (catalog number 2112-28ML) is optimized for light microscopy. Features labeled with 12 nm Nanogold® will be stained black or purple in the light microscope upon silver enhancement. Optimum development times vary among different types of specimens, so different development times should be tried to determine which is best.

Immunolabeling should be completed up to step 6 as described above, then conduct gold enhancement as follows:

1. Wash two times with water.
2. Enhance using GoldEnhance™ LM (4 to 12 minutes).
3. Rinse with deionized water (2 X 5 mins).
4. The specimen may now be stained if desired before examination, with usual reagents.

Immunoblotting

Immunoblotting with Gold Enhancement (recommended)

For more details and illustrations, see our newsletter article on immunoblotting with Nanogold® conjugates and GoldEnhance™ on our web site (https://www.nanoprobes.com/newsletters/Vol8_Iss10.html#2).

Suggested procedure:

REAGENTS AND EQUIPMENT:

- Phosphate buffered saline (PBS): 20 mM sodium phosphate buffer pH 7.4 and 150 mM NaCl.
- Specific antigen (target protein or other biomolecule).
- Nitrocellulose (NC) membrane 0.2 µm pore size.
- Blotting Paper to wick membrane dry.
- Orbital Shaker
- Washing buffer (TBS-Tween 20): 20 mM Tris pH 7.6, with 150 mM NaCl and 0.1 % Tween-20.
- Nonfat dried milk (Carnation)
- GoldEnhance™ EM (Nanoprobes Product No. 2113-8ML) or GoldEnhance™ Blots (Nanoprobes Product No. 2115-48ML).
- Specific Nanogold® antibody conjugate.

PROCEDURE:

Antigen Application:

1. Prepare antigen solutions with a series of dilutions (0.01mg/mL, 0.001mg/mL, 0.0005mg/mL, 0.0001 mg/mL, 0.00005 mg/mL, 0.00001 mg/mL and 0.000005 mg/mL) using PBS, pH7.4.
2. Pipette 1 µL of above solutions to a dry nitrocellulose membrane; prepare 2 duplicates as a negative control.
3. Negative control 1: No antigen, No antibody.
4. Negative control 2: No antigen with NG-conjugate incubation.
5. Air-dry for 30 minutes

Blocking:

6. Immerse membranes in 8 mL of TBS-Tween 20 for 5 minutes.
7. Block membranes in 8 mL of TBS-Tween 20 containing 5 % nonfat dried milk for 30 minutes at room temperature.

Binding of Nanogold® antibody conjugate:

8. Dilute Nanogold® antibody conjugate in TBS-Tween 20 containing 1% nonfat dried milk to 4 µg/mL (1:20 Dilution: 300 µL conjugate + 5.30 ml TBS-gelatin containing 1% nonfat dried milk).
9. Incubate the membranes in 8 mL of diluted conjugate solution for 30 minutes at room temperature.
10. Incubate the control membrane in 8 mL of TBS-Tween20 containing 1% nonfat dried milk for 30 minutes at room temperature.

Autometallographic Detection:

11. Wash membranes three times for 3 min each in 8 mL of TBS-Tween 20. Wash membranes thoroughly in 8 mL of deionized water (4 x 3 minutes). Make sure strips are washed separately according to what they are incubated in (strips incubated in one lot of a conjugate are washed in a separate dish from strips that are incubated in TBS-Tween 20 with 1% nonfat dried milk without conjugate, strips incubated in different lots are washed separately).
12. Perform gold enhancement according to instructions (mix solutions A and B, wait 5 minutes, then add C and D).
13. Record the number of observed spots and time when the spots appear. Record the time when background appears on the control membrane.
14. After 15 minutes, remove the enhancement solution. Rinse membranes with water (3 x 3 minutes) and air-dry for storage.

Tween-20 and nonfat dried milk may also improve the performance of Nanogold® conjugates used with silver enhancement.

Immunoblotting with silver enhancement

The basic procedure for gold immunoblotting has been described by Moeremans et al.[17] For best results, the membrane should be hydrated before use by simmering in gently boiling water for 15 minutes. Best results are obtained when the antigen is applied using a 1 µL capillary tube. The procedure for immunoblots is as follows, if the 12 nm Nanogold® conjugate is the primary antibody:

1. Spot 1 µL dilutions of the antigen in buffer 4 onto hydrated nitrocellulose membrane. Use an antigen concentration range from 100 to 0.01 pg / µL.
2. Block with buffer 1 for 30 minutes at 37°C.
3. Rinse with buffer 1 (3 X 10 mins).
4. Incubate with a 1/100 to 1/200 dilution of the 12 nm Nanogold® reagent in buffer 2 for 2 hours at room temperature.
5. Rinse with buffer 3 (3 X 5 mins), then buffer 4 (2 X 5 mins).
6. OPTIONAL (may improve sensitivity): Postfix with glutaraldehyde, 1% in buffer 4 (10 mins).
7. Rinse with deionized water (2 X 5 mins).
8. OPTIONAL (may reduce background): Rinse with 0.05 M EDTA at pH 4.5 (5 mins).
9. Develop with freshly mixed silver developer for 20-25 minutes or as directed in the instructions for the silver reagent, twice. Rinse thoroughly with deionized water between developments to remove all the reagent.
10. Rinse several times with deionized water.

CAUTION: 12 nm Nanogold® particles may degrade upon exposure to concentrated thiols such as ß-mercaptoethanol or dithiothreitol. If such reagents must be used, concentrations should be kept below 1 mM and exposure restricted to 10 minutes or less.

Buffer 1:
20 mM phosphate
150 mM NaCl
pH 7.4
4% BSA (bovine serum albumin)
2 mM sodium azide (NaN_3)

Buffer 2:
20 mM phosphate
150 mM NaCl
pH 7.4
0.8% BSA
1% normal serum; use serum of the host animal for the 5 nm Nanogold® antibody
0.1% gelatin (Type B, approx. 60 bloom)
Optional, may reduce background:
0.5 M NaCl
0.05% Tween 20

Buffer 3:
20 mM phosphate
150 mM NaCl
pH 7.4
0.8% BSA (bovine serum albumin)
2 mM sodium azide (NaN_3)

Buffer 4 (PBS):
20 mM phosphate
150 mM NaCl
pH 7.4

Other procedures may be used; for example the 12 nm Nanogold® reagent may be used as a tertiary labeled antibody, or a custom 12 nm Nanogold® conjugate may be the primary probe. If additional antibody incubation steps are used, rinse with buffer 3 (3 X 10 mins) after incubation.

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