NANOGOLD®

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PRODUCT INFORMATION

5 NM AZIDO-NANOGOLD® LABELING REAGENT

Product Name:	5 nm Azido-Nanogold®
Catalog Numbers:	2226-5NMOL
Appearance:	Dark red solution
Revision:	2.1 (April 2024)

Congratulations on your acquisition of a revolutionary new gold labeling reagent: 5 nm Azido-Nanogold®. With this biorthogonal reagent, you can label any alkyne-modified molecule of interest, including peptides, proteins, oligonucleotides, and cellular components, with our 5 nm Nanogold® gold nanoparticle for localization and detection.

CONTENTS

Product Information
Thiol Caution
Other Cautions
Introduction to Labeling with 5 nm Nanogold®
Click Reaction Conditions
Characterization of Nanogold® conjugates
General Considerations with Nanogold® Reagents
Using Stains with Nanogold®
Considerations for Viewing Nanogold® in the Electron Microscope
References

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

Nanogold® is a specially developed gold label, prepared using chemically functionalized gold nanoparticles. Unlike conventional colloidal gold reagents which are non-specifically adsorbed to proteins, Nanogold® reagents react selectively and specifically with target chemical groups in the molecule to be labeled,[1] which means that labeling can be targeted or restricted to specific sites within the conjugate biomolecule, This kit contains the 5 nm Nanogold® particle with azido functionalities incorporated into ligands coordinated to the surface of the gold particle. 5 nm Azido-Nanogold® reacts specifically and selectively with alkynes through two reactions:

(a) with a non-strained alkynes to form a 1,2,3-triazole via a 1,3-dipolar cycloaddition catalyzed by copper (I), known as a **c**opper-**c**atalyzed **a**zide-**a**lkyne **c**ycloaddition (**CuAAC**) reaction (Figure **1**);[2,3] and

(b) with a strained alkyne via a 1,3-dipolar cycloaddition reaction known as a **s**train-**p**romoted **a**lkyne **a**zide **c**ycloaddition (**SPAAC**), which does not require a copper (I) catalyst.[4,5]

The advantage of these cycloaddition reactions is that they are biorthogonal: azides and alkynes react selectively only with each other, and do not react with any naturally occurring cellular components. Copper (I), or Cu(I), is generated in situ from Cu(II) using a reducing agent, such as sodium ascorbate in the presence of accelerating ligands such as tris(3-hydroxypropyltriazolymethyl) amine, THPTA.[6] The water-soluble THPTA click ligand binds Cu(I), protects against histidine oxidation, and intercepts reactive oxygen species, affording biological compatibility for Click reactions.[7] The THPTA ligand has been used to label live cells effectively with high efficiency while maintaining cell viability.[8]

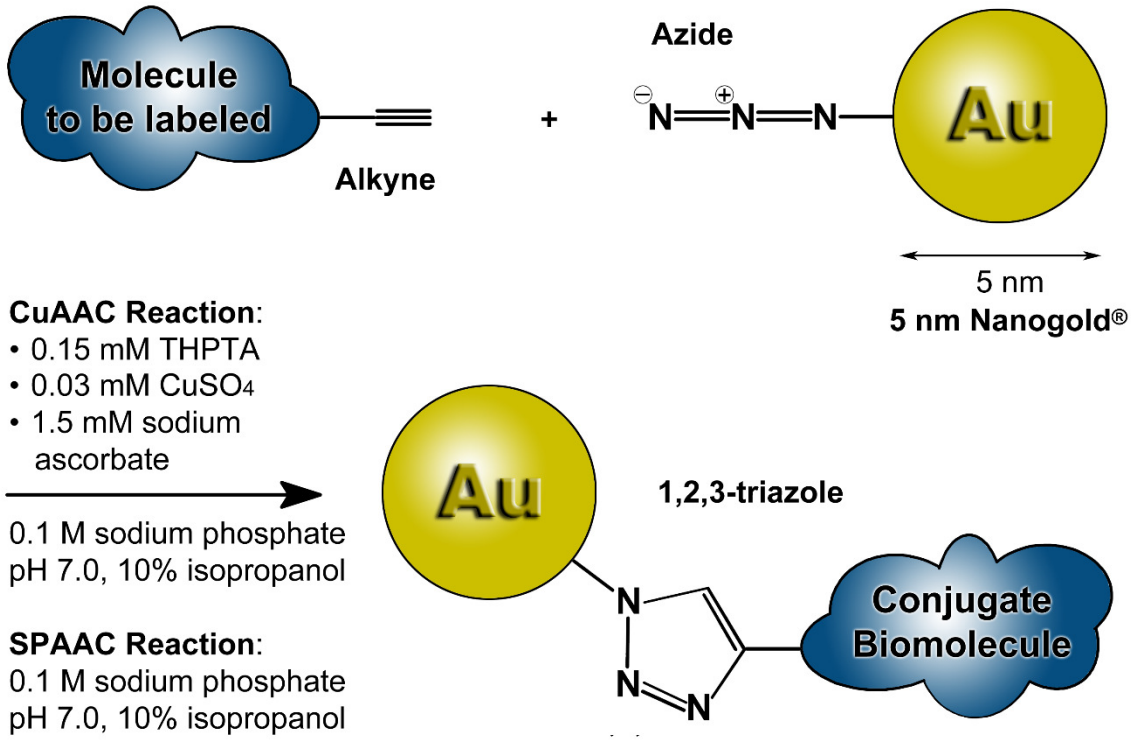

Figure 1: Schematic showing 5 nm Azido-Nanogold® labeling of an alkyne via 1,3-dipolar cycloaddition: (a) CuAAC reaction catalyzed by copper, Cu(I); and (b) SPAAC reaction.

5 nm Azido-Nanogold® is supplied as a solution in 0.1 M sodium phosphate at pH 7.0, at a concentration of 5 nmol/mL (5 x 10^{-6} M, or 5 µM), with optical density (OD) of 60 at the maximum absorption wavelength (λ_{max}) of the plasmon resonance (close to 520 nm). This is approximately sufficient to label about 0.75 mg of IgG using a 1 : 1 Nanogold® : IgG ratio. The labeling reagent should be refrigerated and stored at 2 - 8°C. Nanogold® conjugates are stable to wide ranges of pH and ionic strength, and are not radioactive or carcinogenic. Nanogold®-labeled biomolecules prepared via CuAAC or SPAAC reactions can be detected or localized by electron microscopy or 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.

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

Silver and gold 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.
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/Silver-Enhancers.html (silver enhancement)
https://www.nanoprobes.com/products/GoldEnhance.html (gold enhancement)

THIOL CAUTION

Nanogold® particles may be displaced or degraded upon exposure to thiols such as ß-mercaptoethanol or dithiothreitol. Thiol compounds used in the reduction of protein molecules (or other biomolecules) should be removed from the protein by gel filtration before Nanogold® conjugation. Dialysis does NOT provide acceptable purification in this application. A small amount of residual thiol reagent can severely limit the performance of Nanogold®.

OTHER CAUTIONS

Although Nanogold® is usually stable under demanding conditions, including pH values lower than 4 or ionic strengths above 1 M, Nanogold® reagents labeled specimens or conjugates may not be stable above 50°C for extended period of time, .e.g. over one week, and best results are obtained at room temperature or 4°C. In such cases, incubations at 37°C for extended period of time should be avoided, and the use of low temperature embedding media (e.g., Lowicryl) is recommended if labeling before embedding.[9] It is not recommended to bake tissue blocks with Nanogold®. If your experiment requires higher temperature embedding, then silver or gold enhancement should be performed before embedding.

INTRODUCTION TO LABELING WITH 5 NM NANOGOLD®

General considerations

There are two important differences between our 5 nm Nanogold® reagents and our smaller, 1.4 nm Nanogold® reagents, which should be considered when planning and conducting labeling reactions. Before starting a labeling reaction, you should consider both (1) how to optimize the reaction to maximize your yield of the desired conjugate, and (2) how you can most effectively separate the conjugate from unreacted starting materials and side products.

Specifically:

(1) 5 nm Nanogold® reagents are multi-functional

Unlike the smaller 1.4 nm Nanogold® reagents, each 5 nm gold nanoparticle may contain as many as 20 or more reactive groups. If they are reacted with molecules containing more than one target reactive group, they can form extended oligomers, leading to aggregation or formation of intractable precipitates.

The following strategies will help to minimize this risk:

(a) If possible, use a conjugation reaction that targets a unique functional group. For example, if the molecule you wish to label has several lysine residues (amines) but only one cysteine (thiol), label at the cysteine rather than the amine sites. To use 5 nm Azido-Nanogold®, modify the thiol to introduce a compatible reactivity using an appropriate Click modification reagent: for example, you can use DBCO-PEG4-maleimide to introduce a DBCO group, which reacts selectively with 5 nm Azido-Nanogold®.

(**b**) **Take advantage of immobilization**. If you can immobilize your target before labeling, you will prevent the 5 nm Azido-Nanogold® from linking molecules of the conjugate biomolecule together and ensure more quantitative labeling. If you are using 5 nm Azido-Nanogold® to label a probe that binds a target in live cells or tissues, it may be helpful to allow the probe to bind, then apply the 5 nm Azido-Nanogold®, especially if excess 5 nm Azido-Nanogold® may be easily removed by washing.

(**c**) **Use a reaction stoichiometry that favors the desired ratio of 5 nm Azido-Nanogold® to conjugate biomolecule**:

(i) If you require 1 : 1 labeling and monomeric conjugates: if you are labeling a molecule that has a single reactive group, use a 1 : 1 ratio of 5 nm Azido-Nanogold® : conjugate biomolecule, or a small excess of 5 nm Azido-Nanogold®. This will favor a 1 : 1 ratio, ensure that most or all of the Nanogold® particles are conjugated, while keeping the probability of reaction between a conjugate biomolecule and an already conjugated 5 nm Azido-Nanogold® low. If you are labeling a biomolecule with more than one reactive group, then using an excess of 5 nm Azido-Nanogold® will usually favor the formation of 1 : 1 conjugates, especially if the molecule you are labeling is small and reaction with a second, much larger Nanogold® would be sterically hindered. However, if you are labeling a large biomolecule, then to avoid the formation of extended oligomers, a better approach may be to use an excess of the conjugate biomolecule. The large size of 5 nm Azido-Nanogold® brings additional considerations, as discussed below.

(ii) If you require multiple conjugate biomolecules linked to one 5 nm Azido-Nanogold®: use an excess of the conjugate biomolecule. If your conjugate biomolecule has more than one reactive group, use a large excess to minimize the probability that an already conjugated biomolecule will react with a second Nanogold® to form oligomers. When you are labeling a molecule that is significantly smaller than the 5 nm Nanogold®, using an excess of the conjugate biomolecule will ensure complete conjugation of the Nanogold®, which makes separation easier and provides more options.

Choosing a separation method is discussed in more detail below.

(**2**) **5 nm Nanogold® reagents are large molecules**.

Their overall diameter, including the layer of coordinated organic molecules, is about 17 nm. This is larger than many of the 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 each component fragment is about 6 nm in width. Based upon an average protein density of 1.22 ± 0.02 g/cm^3, a globular protein with the same dimensions as 5 nm Azido-Nanogold®, a 17 nm diameter sphere, would have a molecular weight of 1.89 x 10^6 Da. Thyroglobulin, which is used as s high molecular weight standard for chromatographic separations, has a molecular weight of only 669,000 Da, and occupies a volume about one-third that of 5 nm Azido-Nanogold®. Because proteins usually are not spherically symmetrical, it is reasonable to assume that for separation planning, a 1 x 10^6 Da protein is about the same size as 5 nm Azido-Nanogold®.

This is an important consideration in choosing a method for separating 5 nm Azido-Nanogold® conjugates. If you plan to separate conjugates by gel filtration, the best strategy is to use an excess of the smaller of the two reagents, because it is easier to separate the unreacted excess reagent. The larger reagent is the limiting reagent, and completely reacts. The size difference between the conjugate and excess smaller reagent is greater than that between the conjugate and any unreacted larger reagent, and therefore, their chromatographic resolution will be better.

However, the reaction stoichiometry that gives better separation may conflict with that required to ensure a 1 : 1 ratio of 5 nm Azido-Nanogold® : conjugate biomolecule, or to avoid aggregation or precipitation.

For best results, consider the following strategies:

(**a**) **If you need to use an excess of the larger reagent to avoid aggregation or precipitation, use ammonium sulfate precipitation** rather than gel filtration to separate and purify labeled proteins. This has proven effective in our testing and is not dependent on size. If you are labeling a nucleic acid such as DNA, use ethanol precipitation.

(**b**) **If you are using gel filtration, select your gel carefully**. Only a few gels have molecular weight separation ranges that extend high enough to effectively separate 5 nm Azido-Nanogold® conjugates from excess smaller molecules. In general, we find that agarose gels are more compatible with Nanogold® conjugates than acrylate gels. Superose-6 (Cytiva), which has a MW fractionation range of 5 x 10^4 – 5 x 10^6 Da (exclusion limit 4 x 10^7 Da) effectively separates 5 nm Azido-Nanogold® conjugates, as both conjugates and unconjugated smaller molecules are eluted within the separation range and both are resolved from any larger oligomers or aggregates that are excluded. Other gels with appropriate separation characteristics include the larger molecular weight range Bio-Gel columns

from Bio-Rad: Bio-Gel A-5m gel (MW separation range 10,000–5,000,000 Da), 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).

CLICK REACTION CONDITIONS

EXAMPLE PROTOCOLS

Protocol for Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) reactions

This section contains a general protocol for click reactions using copper-catalyzed azide-alkyne cycloaddition (CuAAC) reactions. This protocol may be used as a starting point for optimization of your particular click chemistry procedures.

1. Prepare a 50 µM solution of the alkyne-modified molecule to be labeled in 0.1 M phosphate pH 7.0 containing 10% DMSO. If possible, use a unique alkyne modification, so that the molecule to be labeled contains only a single alkyne group.

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

   Proteins with more than one alkyne - both separation methods:

   (a) If the protein to be labeled has multiple alkyne modifications and is larger than about 150 kDa (150,000 MW), prepare 20 µL (0.02 mL), or 1 nmol (to give a 5 : 1 ratio of 5 nm Azido-Nanogold® : protein), regardless of separation method. This will help avoid oligomerization.

   (b) If the protein to be labeled has multiple alkyne modifications and is smaller than about 150 kDa (150,000 MW), prepare 50 µL (0.05 mL), or 2.5 nmol (to give a 2 : 1 ratio of 5 nm Azido-Nanogold® : protein), regardless of separation method. This will help avoid oligomerization.

   Proteins with a single unique alkyne – ammonium sulfate precipitation:

   (c) Prepare 100 µL (0.1 mL), or 5 nmol (to give a 1 : 1 ratio of 5 nm Azido-Nanogold® : protein). This should ensure the highest yield of Nanogold® conjugate and the smallest amounts of unreacted starting materials.

   Proteins with a single unique alkyne – gel filtration separation:

   (d) If the protein to be labeled has just one unique alkyne modification and is larger than about 1,000 kDa (1 x 10^6 MW) and you plan to use gel filtration separation, prepare 50 µL (0.05 mL), or 2.5 nmol (to give a 2 : 1 ratio of 5 nm Azido-Nanogold® : protein).

   (e) If the protein to be labeled has just one unique alkyne modification and is between about 1,000 kDa (1 x 10^6 MW) and 150 kDa (150,000 MW), and you plan to use gel filtration separation, prepare 100 µL (0.1 mL), or 5 nmol (to give a 1 : 1 ratio of 5 nm Azido-Nanogold® : protein). This should ensure the highest yield of Nanogold® conjugate and the smallest amounts of unreacted starting materials.

   (f) If the alkyne-modified protein has just one unique alkyne modification and is smaller than 150 kDa (150,000 MW), and you plan to use gel filtration separation, prepare 500 µL (0.5 mL), or 25 nmol (to give a 5 - fold excess of protein over 5 nm Azido-Nanogold®). However, if 1 : 1 conjugate stoichiometry is important, use a smaller excess (1.5-fold is suggested) or 1 : 1 reaction stoichiometry.

   If you are labeling an oligonucleotide and plan to separate the Nanogold®-labeled product by ethanol precipitation, prepare 100 µL, or 5 nmol (to give a 1 : 1 ratio of 5 nm Azido-Nanogold® : alkyne-modified oligonucleotide). This should ensure the highest yield of Nanogold® conjugate and the smallest amounts of unreacted starting materials.

2. Prepare the following click solutions:

   - 15 mM THPTA ligand in water
   - 6 mM $CuSO_4$ in water
   - 300 mM sodium ascorbate in water
   - 5 nm Azido-Nanogold® in 1.0 mL of aqueous 0.1M sodium phosphate buffer at pH 7.0 (as supplied). If necessary, add sufficient isopropanol to make up 10% of the final reaction volume (e.g. 0.2 mL if the reaction volume will be 2.0 mL).

3. Mix 10 µL of 6 mM $CuSO_4$ with 20 µL of 15 mM THPTA in a conical tube.

4. Add the specified amount of 50 µM alkyne-modified molecule to be labeled, prepared in step 1, to 1.0 mL of 5 nm Azido-Nanogold® solution. Vortex.

5. Add the mixture of alkyne-modified molecule to be labeled and 5 nm Azido-Nanogold® to the mixture of $CuSO_4$ and THPTA.

6. Add 10 µL of 300 mM sodium ascorbate to the mixture from Step 4. Vortex. Incubate on a shaker at room temperature for 1 hour.

7. Separate the unbound gold particles from the protein 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 your labeled protein from smaller molecules. Choose a separation matrix with a separation range such that 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 MWCO). Elute with 0.02 M sodium phosphate at pH 7.4 with 150 mM sodium chloride; 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.

The resulting conjugated proteins or nucleic acids in lysate are ready for downstream processing or analysis. See Hong et al[8] for detailed protocol and suggestions for live cell labeling.

Protocol for Strain-Promoted Alkyne Azide Cycloaddition (SPAAC) reactions

This section contains a general protocol for copper-free labeling using strain-promoted alkyne azide cycloaddition (SPAAC) click reactions.[5,10] This protocol may be used as a starting point for optimization of your particular click chemistry procedures.

1. Prepare a 50 µM solution of the alkyne-modified molecule to be labeled in 0.1 M phosphate pH 7.0 containing 10% DMSO. Whenever possible, use a unique alkyne modification, so that the molecule to be labeled contains only a single alkyne group.

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

   Proteins with more than one alkyne - both separation methods:

   (a) If the protein to be labeled has multiple alkyne modifications and is larger than about 150 kDa (150,000 MW), prepare 20 µL (0.02 mL), or 1 nmol (to give a 5 : 1 ratio of 5 nm Azido-Nanogold® : protein), regardless of separation method. This will help avoid oligomerization.

   (b) If the protein to be labeled has multiple alkyne modifications and is smaller than about 150 kDa (150,000 MW), prepare 50 µL (0.05 mL), or 2.5 nmol (to give a 2 : 1 ratio of 5 nm Azido-Nanogold® : protein), regardless of separation method. This will help avoid oligomerization.

Proteins with a single unique alkyne – ammonium sulfate precipitation:

(c) Prepare 100 µL (0.1 mL), or 5 nmol (to give a 1 : 1 ratio of 5 nm Azido-Nanogold® : protein). This should ensure the highest yield of Nanogold® conjugate and the smallest amounts of unreacted starting materials.

Proteins with a single unique alkyne – gel filtration separation:

(d) If the protein to be labeled has just one unique alkyne modification and is larger than about 1,000 kDa (1 x 10^6 MW) and you plan to use gel filtration separation, prepare 50 µL (0.05 mL), or 2.5 nmol (to give a 2 : 1 ratio of 5 nm Azido-Nanogold® : protein).

(e) If the protein to be labeled has just one unique alkyne modification and is between about 1,000 kDa (1 x 10^6 MW) and 150 kDa (150,000 MW), and you plan to use gel filtration separation, prepare 100 µL (0.1 mL), or 5 nmol (to give a 1 : 1 ratio of 5 nm Azido-Nanogold® : protein). This should ensure the highest yield of Nanogold® conjugate and the smallest amounts of unreacted starting materials.

(f) If the alkyne-modified protein has just one unique alkyne modification and is smaller than 150 kDa (150,000 MW), and you plan to use gel filtration separation, prepare 500 µL (0.5 mL), or 25 nmol (to give a 5 - fold excess of protein over 5 nm Azido-Nanogold®). However, if 1 : 1 conjugate stoichiometry is important, use a smaller excess (1.5-fold is suggested) or 1 : 1 reaction stoichiometry.

If you are labeling an oligonucleotide and plan to separate the Nanogold®-labeled product by ethanol precipitation, prepare 100 µL, or 5 nmol (to give a 1 : 1 ratio of 5 nm Azido-Nanogold® : alkyne-modified oligonucleotide). This should ensure the highest yield of Nanogold® conjugate and the smallest amounts of unreacted starting materials.

2. Add the the specified amount of 50 µM alkyne-modified molecule to be labeled, prepared in step 1, to the 0.5 mL of 5 nm Azido-Nanogold® solution. Vortex the mixture to ensure thorough solution and mixing.

3. Incubate the reaction mixture, either

   (a) on a shaker at room temperature for 1 hour, or

   (b) at 2 – 8°C overnight in the refrigerator

4. Separate the unbound gold particles from the protein 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 your labeled protein from smaller molecules. Choose a separation matrix with a separation range such that 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 MWCO). Elute with 0.02 M sodium phosphate at pH 7.4 with 150 mM sodium chloride; 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.

The resulting conjugated proteins or nucleic acids in lysate are ready for downstream processing or analysis. See Hong et al[8] for detailed protocol and suggestions for live cell labeling.

CHARACTERIZATION OF NANOGOLD® CONJUGATES

The purified Nanogold® conjugated peptide, protein or oligonucleotide is normally red colored at a high concentration, and can be detected by UV-Vis spectroscopy in the wavelength range 250 - 800 nm. Unlike the UV-Vis spectrum of unlabeled peptide, protein or oligonucleotide, which is usually near or at baseline from 300 - 800 nm, the absorption spectra of Nanogold® conjugates have significant absorption over the range of 300 - 800 nm, with an absorption maximum (λ_{max}) close to 520 nm (the plasmon resonance).

Estimation of Degree of Labeling (DOL): To calculate the amount of 5 nm Nanogold®, measure the absorption at the λ_{max} given in the Product Specifications, and at either 280 nm (for proteins) or 260 nm (for oligonucleotides). Use the absorbance at λ_{max} to calculate the molar concentration of 5 nm Nanogold®, using the molar extinction coefficient of 1.2×10^7 $M^{-1}cm^{-1}$. Next, calculate the contribution from the 5 nm Nanogold® to the absorbance at 280 nm (for proteins) or 260 nm (for oligonucleotides). To do this, multiply the observed absorbance at λ_{max} by the appropriate ratio of the Optical Densities given in the Product Specifications: $\gamma_{gold\ (280nm/\lambda max)}$ ($OD_{280nm}/OD\lambda_{max}$) for proteins, or $\gamma_{gold\ (260nm/\lambda max)}$ ($OD_{260nm}/OD\lambda_{max}$) for oligonucleotides. Subtract the absorption due to Nanogold® from the measured absorbance at 280 nm; the result is the absorbance of the protein or oligonucleotide. Divide this result by the extinction coefficient at 280 nm (ε_{280nm}) or 260 nm (ε_{260nm}) to calculate the molar concentration of the protein or oligonucleotide respectively.

$$[\text{Nanogold}^{®}] = [A_{\lambda max}]/1.2 \times 10^7$$

$$[\text{Protein}] = [A_{280nm} - \gamma_{gold\ (280nm/\lambda max)} \times A_{\lambda max}]/\varepsilon_{protein\ at\ 280\ nm}$$

or

$$[\text{Nucleic Acid}] = [A_{280nm} - \gamma_{gold\ (280nm/\lambda max)} \times A_{\lambda max}]/\varepsilon_{oligonucleotide\ at\ 260\ nm}$$

$$\text{DOL} = [\text{Nanogold}^{®}]/[\text{Protein}]$$

or

$$\text{DOL} = [\text{Nanogold}^{®}]/[\text{oligonucleotide}]$$

Characterization by Gel Electrophoresis: Purified Nanogold® conjugates or 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 Nanogold® conjugate, or Nanogold® conjugate mixture with unlabeled peptide, protein or oligonucleotide and unreacted 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. Nanogold® has a molecular weight of about 15,000 dalton and negligible charge, and contributes little to the charges of labeled peptides, proteins or polynucleotides. **Caution**: Nanogold® conjugates and Nanogold® reagents should not be heated with ß-mercaptoethanol before loading onto gels as ß-mercaptoethanol degrades 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. Wash with deionized water for 4 x 5 minutes and continue overnight. The Nanogold® conjugate and Nanogold® reagent bands will become brown in color upon incubation with LI Silver.

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

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

GENERAL CONSIDERATIONS FOR USING 5 NM AZIDO-NANOGOLD® REAGENTS

- Nanogold® is an extremely uniform 5 nm diameter gold particle (±10%).

- 5 nm Azido-Nanogold® reacts with an alkyne to form a 1, 2, 3-triazole conjugate in CuAAC.
- Nanogold® is covalently attached to the peptide, protein, polycucleotide or live cells after click reactions
- Nanogold® conjugates contain no aggregates. This is in sharp contrast to other colloidal gold conjugates that usually are prepared by centrifugation to remove the largest aggregates and frequently contain smaller aggregates.
- Nanogold® particles do not have affinity to proteins as do colloidal golds. This reduces background and false labeling.
- 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 NANOGOLD®

5 nm Nanogold® particles are relatively small, and in thick sections or particularly dense tissue, over staining with OsO_4, uranyl acetate or lead citrate may tend to obscure direct visualization of individual Nanogold® particles. Three recommendations for improved visibility of Nanogold® are:

1. Use of reduced amounts or concentrations of usual stains.
2. Use of lower atomic number stains such as Nanoprobes NanoVan™, a Vanadium based stain.[12]
3. Enhancement of Nanogold® using silver or gold enhancers.

SPECIAL CONSIDERATIONS FOR DIRECT VIEWING OF NANOGOLD® IN THE ELECTRON MICROSCOPE

For most work, silver or gold enhancement is recommended to give a good signal in the electron microscope (see below). For applications where direct visualization of the Nanogold® may be desirable, thin samples are recommended and caution is advised when using other stains.

Nanogold® provides improved resolution and smaller probe size over colloidal gold antibody products. Since Nanogold® is only 1.4 nm in diameter, it will not only be smaller, but will appear less intense than, for example, a 10 nm gold particle for applications such as post-embedding labeling. With careful work, however, Nanogold® may be seen directly through the binoculars of a standard EM in many types of specimens. However, achieving the high resolution necessary for this work may require modifications to your equipment or technique. Several suggestions follow:

1. Before you start a project with Nanogold® it is helpful to see it so you know what to look for. Dilute the Nanogold® stock 1:5 and apply 4 µl to a grid for 1 minute. Wick the drop and wash with deionized water 4 times.
2. View Nanogold® at 100,000 X magnification with 10 X binoculars for a final magnification of 1,000,000 X. Turn the emission up full and adjust the condenser for maximum illumination.
3. The alignment of the microscope should be in order to give 0.3 nm resolution. Although the scope should be well aligned, you may be able to skip this step if you do step 4.
4. Objective stigmators should be optimally set at 100,000 X. Even if the rest of the microscope optics are not perfectly aligned, adjustment of the objective stigmators may compensate and give the required resolution. You may want to follow your local protocol for this alignment but since it is important, a brief protocol is given here:
    a. At 100,000 X (1 X 10^6 with binoculars), over focus, under focus, then set the objective lens to in focus. This is where there is the least amount of detail seen.
    b. Adjust each objective stigmator to give the least amount of detail in the image.
    c. Repeat steps a and b until the in focus image contains virtually no contrast, no wormy details, and gives a flat featureless image.

5. Now underfocus slightly, move to a fresh area, and you should see small black dots of 1.4 nm size. This is the Nanogold®. For the 1:5 dilution suggested, there should be about 5 to 10 gold spots on the small viewing screen used with the binoculars. Contrast and visibility of the gold particles is best at 0.2 - 0.5 m defocus, and is much worse at typical defocus values of 1.5 - 2.0 m commonly used for protein molecular imaging.

6. In order to operate at high magnification with high beam current, thin carbon film over fenestrated holey film is recommended. Alternatively, thin carbon or 0.2% Formvar over a 1000 mesh grid is acceptable. Many plastic supports are unstable under these conditions of high magnification/high beam current and carbon is therefore preferred. Contrast is best using thinner films and thinner sections.

7. Once you have seen Nanogold® you may now be able to reduce the beam current and obtain better images on film. For direct viewing with the binoculars reduction in magnification from 1,000,000 X to 50,000 X makes the Nanogold® much more difficult to observe and not all of the golds are discernable. At 30,000 X (300,000 X with 10 X binoculars) Nanogold® particles are not visible. It is recommended to view at 1,000,000 X, with maximum beam current, align the objective stigmators, and then move to a fresh area, reduce the beam, and record on film.

8. If the demands of high resolution are too taxing or your sample has an interfering stain, a very good result may be obtained using silver or gold enhancement to give particles easily seen at lower magnification.

REFERENCES

1. (a) Hainfeld, J. F., and Powell R. D.: New Frontiers in Gold Labeling. *J Histochem Cytochem.*, 48, 471-80 (2000). PMID: 10727288. DOI: 10.1177/002215540004800404.

   (b) Furuya, F. R., and Hainfeld, J. F.: A 1.4-nm gold cluster covalently attached to antibodies improves immunolabeling. *J. Histochem. Cytochem.*, **40**, 177 (1992). PMID: 1552162. DOI: 10.1177/40.2.1552162.

   (c) Furuya, F. R.; Hainfeld, J. F., and Powell, R. D.: A new 1.4 nm Gold-Fab' Probe. *Proc. 49th Ann. Mtg., Electron.Micros. Soc. Amer.*; Bailey, G. W. (Ed.), San Francisco Press, San Francisco, CA; **1991**, p. 284-285. (https://www.nanoprobes.com/applications/MSA92ng.html).

2. Rostovtsev, V.; Green, L.; Fokin, V., and Sharpless, K.: A stepwise Huisgen cycloaddition process: copper (I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem. Int. Ed.*, **41**, No.14, 2596 -2599 (2002). PMID: 12203546. DOI: 10.1002/1521-3773(20020715)41:14<2596::AID-ANIE2596>3.0.CO;2-4.

3. Tornϕe, C.; Christensen, C., and Meldal, M.: Peptidotrazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1, 3 -dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem.*, **67,** 3057-3064 (2002). PMID: 11975567. DOI: 10.1021/jo011148j.

4. Jewett, J. C., and Bertozzi, C. R.: Cu-free click cycloaddition reactions in chemical biology. *Chem. Soc. Rev.*, **39**,1272-1279 (2010). PMID: 20349533. PMCID: PMC2865253. DOI: 10.1039/b901970g.

5. Jang, S.; Sachin, K.; Lee, H.-J.; Kim, D. W., and Lee, H. S.: Development of a simple method for protein conjugation by copper-free click reaction and its application to antibody-free Western blot analysis. *Bioconjug. Chem.*, **23**, 2256-2261 (2012). PMID: 23039792. DOI: 10.1021/bc300364z.

6. Presolski, S. I.; Hong, V. P., and Finn, M. G.: Copper-catalyzed azide-alkyne click chemistry for bioconjugation. *Current Protocols in Chemical Biology.*, **3(4)**, 153-162 (2011). DOI: 10.1002/9780470559277.ch110148.

7. Hong, V.; Presolski, S., and Finn, M.: Analysis and optimization of copper-catalyzed azide-alkyne cycloaddition for bioconjugation. *Angew. Chem. Int. Ed.*, **48**, 9879-9883 (2009). PMID: 19943299. PMCID: PMC3410708. DOI: 10.1002/anie.200905087.

8. Hong, V.; Steinmetz, N. F.; Manchester, M., and Finn, M. G.: Labeling live cells by copper-catalyzed alkyne-azide click chemistry. *Bioconjugate. Chem..*, **21(10)**, 1912-1916 (2010). PMID: 20886827. PMCID: PMC3014321. DOI: 10.1021/bc100272z.

9. Krenács, T., and Krenács, L.: Comparison of Embedding Media for Immunogold-Silver Staining. In: *Immunogold-Silver Staining: Principles, Methods and Applications* (M. A. Hayat, Ed.), CRC Press, Boca raton, FL., **1995**: pp. 57-69.

10. Gong, H.; Holcomb, I.; Ooi, A.; Wang, X.; Majonis, D.; Unger, M. A., and Ramakrishnan, R.: Simple Method To Prepare Oligonucleotide-Conjugated Antibodies and Its Application in Multiplex Protein Detection in Single Cells. *Bioconjug. Chem.*, **27**, 217-225 (2016). PMID: 26689321. DOI: 10.1021/acs.bioconjchem.5b00613.

11. Baskin, J. M.; Prescher, J. A.; Laughlin, S. T.; Agard, N. J.; Chang, P. V.; Miller, I. A.; Lo, A.; Codelli, J. A., and Bertozzi, C. R.: Copper-free click chemistry for dynamic in vivo imaging. Proc. Natl. Acad. Sci. U S A, 104, 16793-16797. doi: 10.1073/pnas.0707090104. Epub 2007 Oct 17. PMID: 17942682. PMCID: PMC2040404. DOI: 10.1073/pnas.0707090104.

12. (a) Tracz, E., Dickson, D. W., Hainfeld, J. F., and Ksiezak-Reding, H.: Paired helical filaments in corticobasal degeneration: the fine fibrillary structure with NanoVan. *Brain Res.*, **773**, 33-44 (1997). PMID: 9409702. DOI: 10.1016/s0006-8993(97)00830-5.

    (b) Gregori, L., Hainfeld, J. F., Simon, M. N., and Goldgaber, D. Binding of amyloid beta protein to the 20S proteasome. *J. Biol. Chem.*, **272**, 58-62 (1997). PMID: 8995227. DOI: 10.1074/jbc.272.1.58.

    (c) Hainfeld, J. F.; Safer, D.; Wall, J. S.; Simon, M. N.; Lin, B. J., and Powell, R. D.: Methylamine Vanadate (Nanovan) Negative Stain. *Proceedings of the Fifty-Second Annual Meeting, Microscopy Society of America*; Bailey, G. W., and Garratt-Reed, A. J., Eds.; San Francisco Press, San Francisco, CA, 1994, pp. 132-133. (https://www.nanoprobes.com/applications/MSANV.html).

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