NANOGOLD®

Nanoprobes

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PRODUCT INFORMATION

NANOGOLD®-STREPTAVIDIN

Product Name:	NANOGOLD-Streptavidin conjugate
Catalog Number:	2016
Appearance:	Pale brown solution
Revision:	1.5 (March 2000)

Congratulations on your acquisition of a revolutionary new gold immunoreagent: NANOGOLD®. It is the smallest gold immunoprobe commercially available and is produced very differently from colloidal gold preparations. It therefore has many superior features (see below) which can improve the resolution and quality of your research.

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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 newly developed gold immunoprobe. NANOGOLD® reagents consist of affinity-purified Streptavidin, Fab' fragments and IgG antibodies (goat anti-mouse IgG, goat anti-mouse Fab', goat anti-rabbit IgG, goat anti-rabbit Fab', rabbit anti-goat IgG, rabbit anti-goat Fab', and a range of other polyclonal IgG antibodies and Fab' fragments) covalently conjugated to the 1.4 nm NANOGOLD® gold cluster label. NANOGOLD® can be used in immunoblotting, light microscopy, and electron microscopy to provide clear visibility. Stable to wide ranges of pH and ionic strength, this gold immunoprobe is 1.4 nm in diameter. It is not

radioactive or carcinogenic. NANOGOLD® reagents are stored in 20 mM phosphate buffered saline (150 mM NaCl) at pH 7.4, with 0.1 % BSA and 0.05 5 sodium azide as preservatives. NANOGOLD® conjugates should be stored at 2-8°C. DO NOT FREEZE.

GENERAL CONSIDERATIONS FOR IMMUNOSTAINING WITH NANOGOLD® REAGENTS

Basically, normal methodologies may be used successfully with NANOGOLD® immunoreagents. The concentration of antibody and gold is similar to other commercial preparations of colloidal gold antibodies. Therefore similar dilutions and blocking agents are appropriate.

The major difference will be in the results:

NANOGOLD® is an extremely uniform 1.4 nm diameter gold particle (±10%).

Streptavidin-NANOGOLD® is the smallest gold immunoprobe commercially available and will penetrate and reach antigens inaccessible to other gold probes.

Streptavidin - Nanogold® is chromatographically purified through gel filtration columns. There are absolutely no aggregates or other molecular weight impurities. 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.

Close to 1 NANOGOLD® particle to 1 Streptavidin make this product distinct from the 0.2 - 10 variable stoichiometry of other colloidal gold antibody preparations.

NANOGOLD® particles do not have affinity to proteins as do other other colloidal golds. This reduces background and false labeling.

NANOGOLD® develops better with silver than do most other colloidal golds, giving it higher sensitivity. Silver enhancement can be used to make the immunolabeling useful for electron microscopy, light microscopy, and immunoblotting with improved results.

USING STAINS WITH NANOGOLD®

Because the 1.4 nm NANOGOLD® particles are so small, 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 NANOVAN™, a Vanadium based stain.[2]
3. Enhancement of NANOGOLD® with silver developers, such as LI SILVER or HQ SILVER.

TEMPERATURE CAUTION

Although NANOGOLD® is usually stable,[3] under some conditions labeled specimens or conjugates may not be stable above 50°C. Best results are obtained at room temperature or 4°C. Avoid 37°C incubations. Use low temperature embedding media (e.g., Lowicryl) if labeling before embedding;[4] do not bake tissue blocks with NANOGOLD®. If your experiment requires higher temperature embedding, then silver enhancement should be performed before embedding.

THIOL CAUTION

NANOGOLD® particles 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.

IN SITU HYBRIDIZATION WITH NANOGOLD®-STREPTAVIDIN

Procedure (adapted from that of Hacker, G.W., et al.):[5]

Practical considerations: This is a robust and reliable technique for routine use. It is intended for biotinylated hybridization probes. Other types of reporter molecules may be demonstrated by application of a biotinylated linking antibody system. The sensitivity of Nanogold-silver ISH depends, to a large degree, at the dilution of streptavidin-Nanogold and the duration of silver development applied. Careful adjustment of the protease predigestion is necessary. In some preparations, some degree of unwanted background staining in connective tissue is obtained. This is in part due to fixation and possible excessive protease treatment and can often be reduced by application of higher dilutions of streptavidin-Nanogold.

1. Deparaffinize sections from formaldehyde-fixed tissue in fresh xylene (2 times 15 min each).
2. Rinse and rehydrate in graded alcohols and distilled water (2-3 min each).
3. Soak in phosphate-buffered saline (PBS, 20mM, pH 7.6) for 3 min.
4. Incubate with 0.1 mg/mL proteinase K (code no. 1 373 196, from Boehringer Mannheim, Mannheim, FRG) in PBS at 37 °C for about 8 min. The duration is critical and has to be tested very carefully, depending on tissue, fixation and other factors.
5. Rinse in 2 changes of PBS, 3 min.
6. Permeabilize with 0.3% Triton X-100 in PBS for 15 min.
7. Wash in PBS for 2 min.
8. Rinse in 2 changes of distilled water, dehydrate with graded alcohols (50%, 70%, 98% isopropanol) for 1 min each and air-dry the sections.
9. Prehybridize with 1:1 mixture of deionized formamide and 20% dextran sulfate in 2X SSC at 50 °C for 5 min.
10. Carefully shake off excess prehybridization block.
11. Add one drop of biotinylated DNA probe on the section and cover with a small coverslip. Avoid air bubbles.
12. Heat sections on heating block at 92-94 °C for 8-10 min to denature DNA.
13. Incubate in a moist chamber at 37°C overnight (or for at least 2 hours).
14. Post-hybridization washes (5 min each): 2 changes of 4X SSC (1st wash to remove coverslips), 2X SSC, 0.1X SSC, 0.05X SSC, and then distilled water.
15. Put slides into Lugol's iodine solution (Merck, Darmstadt, Germany) for 5 min.
16. Wash in tap water and then distilled water.
17. Put into 2.5% sodium thiosulfate for a few seconds until sections are colorless. Then wash in tap water for 5 min and distilled water for 2 min.
18. Immerse in PBS containing 0.1% fish gelatin (45% concentrate – Cat. No. G-7765, Sigma-Aldrich, Steinheim, Germany) and 0.1% Tween-20 for 5 min.
19. Incubate sections with streptavidin-Nanogold® diluted 1:200 to 1:500 in PBS containing 1% BSA at room temperature for 60 min.
20. Wash in 3 changes of PBS containing 0.1% fish gelatin and 0.1% Tween-20 for 5 min each.
21. Repeatedly wash in distilled water for at least 10 min altogether, the last 2 rinses in ultrapure water (EM-grade).
22. Perform silver acetate autometallography or GoldEnhance development (Nanoprobes, see Protocol 4).
23. Rinse carefully in tap water for at least 3 min. After silver amplification, sections can be counterstained with Nuclear Fast Red, dehydrated and mounted in Permount or in DPX (BDH Chemicals, Poole, UK). Do not use Eukitt.

Solutions:

Phosphate-buffered saline (PBS): 10X PBS (Mg2+ and Ca2+-free) pH 7.6: 11.36 g Na2HPO4, 2.72 g KH2PO4, 87.0 g NaCl in 800 mL distilled water. Adjust pH with concentrated NaOH and add distilled water to a final volume of 1 L.

Standard Sodium Citrate Buffer (SSC): 175.32 g NaCl and 88.23 g sodium citrate in 800 ml distilled water. Adjust pH with NaOH to 7.0 and add distilled water to a final volume of 1 L.

Silver Development:

1. Silver amplification: Place the slides vertically in a glass container (preferably with about 80 mL volume and up to 19 slides; Schiefferdecker-type) and cover them with the mixture of solutions A and B. Staining intensity can be checked in the light microscope during the amplification process, which usually takes about 5-20 min, depending on primary

antibody or nucleic acid probe concentration, incubation conditions, and the amount of accessible antigen or nucleic acid sequence in question.
2. Stop enhancement by washing in distilled water (several changes).
3. After stopping the enhancement process, slides can be examined in a light microscope more carefully. If staining intensity is still too low, wash slides for one more time in double-distilled water and develop further in enhancement solution.

Reagents (Solutions A and B should be freshly prepared for every run):

Solution A: Dissolve 80 mg silver acetate (code 85140; Fluka, Buchs, Switzerland) in 40 mL of glass double-distilled water. (Silver acetate crystals can be dissolved by continuous stirring within about 15 min.)

Citrate buffer: Dissolve 23.5 g of trisodium citrate dihydrate and 25.5 g citric acid monohydrate in 850 mL or deionized or distilled water. This buffer can be kept at 4°C for at least 2-3 weeks. Before use, adjust to pH 3.8 with citric acid solution.

Solution B: Dissolve 200 mg hydroquinone in 40 mL citrate buffer.

Enhancement solution: Just before use, mix solution A with solution B.

Alternative autometallography procedure (using LI Silver, Nanoprobes):

1. Allow LI Silver to come to room temperature.
2. Mix equal amounts of A and B solutions of LI Silver (red and blue capped bottles).
3. Immediately cover sections with developer.
4. Keep in subdued light, but check sections occasionally, using eye or light microscope. Development usually takes 20 min to 45 min.
5. Stop development by rinsing in deionized water. Alternatively, a fixer may be used to stop development immediately. Use a photographic fixer (e.g., Agefix, Agfa Gevaert, FRG, diluted 1:20) (can be reused). Treat for ~1 min. Another alternative is a 2.5% aqueous solution of sodium thiosulfate.
6. If sections are overdeveloped, they may be "back-developed" by 0.2 % Farmer's solution (9 parts sodium thiosulfate + 1 part potassium ferricyanide; form by mixing 0.18 g $Na_2S_2O_3$. 5 H_2O + 0.02 g $K_3Fe(CN)_6$ in 100 ml water). Reversal may be halted with water.
7. Rinse slides carefully in tap water for at least 3 min. After silver amplification, sections can be counterstained with hematoxylin and eosin or nuclear fast red, dehydrated, and mounted in DPX (BDH Chemicals, UK).

ELECTRON MICROSCOPY IMMUNOLABELING WITH NANOGOLD®

If aldehyde-containing reagents have been used for fixation, these must be quenched before labeling. 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. Place 50 - 200 µl of cells into Eppendorf tube and add 5 - 10 µl of primary antibody (or antiserum). Incubate 30 minutes with occasional shaking (do not create bubbles which will denature proteins).
4. Wash cells using PBS-BSA as described in step 1 (2 X 5 mins). Resuspend in 1 ml Buffer 1.
5. Dilute NANOGOLD® ~ 50 times in PBS-BSA buffer and add 30 µl to cells; incubate for 30 minutes with occasional shaking.
6. Wash cells in PBS-BSA as described in step 1 (2 X 5 mins).
7. 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).

CAUTION: NANOGOLD® particles 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.

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

Other procedures may be used; for example the NANOGOLD® reagent may be used as a tertiary labeled antibody in a system where a biotinylated secondary antibody is used with an unlabeled primary antibody. If additional antibody incubation steps are used, rinse with PBS-BSA (3 X 5 mins) after incubation.

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. NANOVAN™ negative stain is specially formulated for use with NANOGOLD® reagents; it is based on vanadium, which gives a lighter stain than uranium, lead or tungsten-based negative stains and allows easier visualization of NANOGOLD® particles with little or no silver enhancement.

Thin Sections

Labeling with NANOGOLD® may be performed before or after embedding.[6,7] Labeling before embedding and sectioning (the pre-embedding method)[6,7] 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)[6,7] 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.

PROCEDURE FOR PRE-EMBEDDING METHOD:[6]

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. Incubate with primary antibody, diluted at usual working concentration in PBS-BSA (30 mins - 1 hour, or usual time. Buffer formulations are given below)
4. Rinse with PBS-BSA (3 X 1 min).
5. Incubate with NANOGOLD® reagent diluted 1/40 - 1/200 in PBS-BSA with 1 % normal serum (usually normal goat serum, or other serum not targeted by antibodies used in earlier steps), for 10 minutes to 1 hour at room temperature.
6. Rinse with PBS-BSA (3 X 1 min), then PBS (3 X 1 min).
7. Postfix with 1 % glutaraldehyde in PBS (10 mins).
8. Rinse in deionized water (2 X 5 min).
9. Dehydrate and embed according to usual procedure. Use of a low-temperature resin (eg. Lowicryl) is recommended; if higher tyemperature embedding resins are used, silver enhancement should be performed before embedding.
10. 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:[6]

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. Incubate with primary antibody, diluted at usual working concentration in PBS-BSA (1 hour or usual time. Buffer formulations are given below)
4. Rinse with PBS-BSA (3 X 1 min).
5. Incubate with NANOGOLD® reagent diluted 1/40 - 1/200 in PBS-BSA with 1 % normal serum (usually normal goat serum, or other serum not targeted by antibodies used in earlier steps), for 10 minutes to 1 hour at room temperature.
6. Rinse with PBS (3 X 1 min).
7. Postfix with 1 % glutaraldehyde in PBS at room temperature (3 mins).
8. Rinse in deionized water for (2 X 5 min).
9. If desired, contrast sections with uranyl acetate and/or lead citrate before examination.

Silver enhancement may also be used to render the NANOGOLD® particles more easily visible (see below); this is recommended if stains such as uranyl acetate or lead citrate are applied. Silver enhancement should be completed before these stains 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

Other procedures may be used; for example the NANOGOLD® reagent may be used as a tertiary labeled probe in a system where a biotinylated secondary antibody is used with an unlabeled primary antibody. If additional antibody incubation steps are used, rinse with PBS-BSA (3 X 5 mins) after incubation.

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

For most work, silver enhancement is recommended to give a good signal in the electron microscope (see below). For particular applications, visualization of the NANOGOLD® directly may be desirable. Generally this requires very thin sampl;es and precludes the use of other stains.

NANOGOLD® provides a much improved resolution and smaller probe size over other colloidal gold antibody products. However, because NANOGOLD® is only 1.4 nm in diameter, it will not only be smaller, but will appear less intense than, for example, a 5 nm gold particle. With careful work, however, NANOGOLD® may be seen directly through the binoculars of a standard EM even in 80 nm thin sections. However, achieving the high resolution necessary for this work may require new demands on your equipment and 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 must 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 clusters 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 enhancement to give particles easily seen at lower magnification.

SILVER ENHANCEMENT OF NANOGOLD® FOR EM

NANOGOLD® will nucleate silver deposition resulting in a dense particle 2-80 nm in size or larger depending on development time. If specimens are to be embedded, silver enhancement is usually performed after embedding, although it may be done first. It 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 NANOGOLD® reagents, low-temperature resins (eg Lowicryl) should be used and the specimens kept at or below room temperature until after silver development has been completed. Silver development is recommended for applications of NANOGOLD® in which these stains are to be used, otherwise the NANOGOLD® particles may be difficult to visualize against the stain.

Our LI SILVER silver enhancement system is convenient and not light sensitive, and suitable for all applications. Improved results in the EM may be obtained using HQ SILVER, which is formulated to give more controllable particle growth and uniform particle size distribution, at neutral pH for best possible ultrastructural preservation.[8]

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. NANOGOLD® will nucleate silver deposition resulting in a dense particle 2-20 nm in size or larger depending on development time. Use nickel grids (not copper).

The relevent procedure for immunolabeling should be followed up to step 7 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 4 min. 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. Alternatively, use of 0.1 % osmium tetroxide instead of 1 % has been found to give similar levels of staining while greatly reducing etching of the silver particles.

NOTE: Treatment with osmium tetroxide followed by uranyl acetate staining can lead to much more drastic loss of the silver enhanced NANOGOLD® particles. This may be prevented by gold toning:[9]

1. After silver enhancement, wash thoroughly with dionized 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.

IMMUNOLABELING AND SILVER ENHANCEMENT WITH NANOGOLD® FOR LIGHT MICROSCOPY

Features labeled with 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 procedure for immunolabeling is similar to that for EM; a suitable procedure 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 components of buffer solutions. The procedure for immunolabeling with NANOGOLD® and silver enhancement is given below.

1. Spin cells onto slides using Cytospin, or use parafin section.
2. Incubate with 1 % solution of bovine serum albumin in PBS (PBS-BSA) for 10 minutes to block non-specific protein binding sites.
3. Incubate with primary antibody, diluted at usual working concentration in PBS-BSA (1 hour or usual time)
4. Rinse with PBS-BSA (3 X 2 min).
5. Incubate with NANOGOLD® reagent diluted 1/40 - 1/200 in PBS-BSA with 1 % normal serum (usually normal goat serum, or other serum not targeted by antibodies used in earlier steps), for 1 hour at room temperature.
6. Rinse with PBS (3 X 5 min).
7. Postfix with 1 % glutaraldehyde in PBS at room temperature (3 mins).
8. Rinse with deionized water (3 X 1 min).
9. OPTIONAL (may reduce background): Rinse with 0.02 M sodium citrate buffer, pH 7.0 (3 X 5 mins).
10. 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.
11. Rinse with deionized water (2 X 5 mins).
12. 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

PBS Buffer:

20 mM phosphate
150 mM NaCl
pH 7.4

0.1% gelatin (high purity)

Optional, may reduce background:

0.5 M NaCl
0.05% Tween 20

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

Other procedures may be used; for example the NANOGOLD® reagent may be used as a tertiary labeled antibody in a system where a biotinylated secondary antibody is used with an unlabeled primary antibody. If additional antibody incubation steps are used, rinse with PBS-BSA (3 X 5 mins) after incubation.

IMMUNOBLOTTING

The basic procedure for gold immunoblotting has been described by Moeremans et al[10], which may be followed. 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:

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. Incubate with primary antibody according to usual procedure (usually 1 or 2 hours).
4. Rinse with buffer 1 (3 X 10 mins).
5. Incubate with a 1/100 to 1/200 dilution of the NANOGOLD® reagent in buffer 2 for 2 hours at room temperature.
6. Rinse with buffer 3 (3 X 5 mins), then buffer 4 (2 X 5 mins).
7. OPTIONAL (may improve sensitivity): Postfix with glutaraldehyde, 1 % in buffer 4 (10 mins).
8. Rinse with deionized water (2 X 5 mins).
9. OPTIONAL (may reduce background): Rinse with 0.05 M EDTA at pH 4.5 (5 mins).
10. 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.
11. Rinse several times with deionized water.

CAUTION: NANOGOLD® particles 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 (normal goat serum, or other serum not targeted by antibodies used in earlier steps)
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, NANOGOLD® - Streptavidin may be used as a tertiary labeled antibody, or a custom NANOGOLD® conjugate may be the primary antibody. If additional antibody incubation steps are used, rinse with buffer 3 (3 X 10 mins) after incubation.

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