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Toward an ICPMS-Linked DNA Assay Based on Gold Nanoparticles Immunoconnected through Peptide Sequences

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ABSTRACT.

Gold nanoparticles modified with anti-mouse IgG have been used to trace oligonucleotides carrying a c-myc peptide. Two strategies, a dot-blot format as well as inductively coupled plasma mass spectrometry (ICPMS) have been used to detect the nanoparticle tracer. For both cases, oligonucleotide-peptide conjugates were first applied to a nitrocellulose membrane using a manifold attached to a suction device. After immobilization of the oligonucleotide by UV radiation, the samples were incubated with an anti-c-myc monoclonal antibody. In the case of the dot-blot format strategy, it was followed by incubation with a secondary antibody conjugated to horseradish peroxidase and development with luminol as chemiluminescent substrate. In the case of ICPMS strategy, it was followed by incubation with the secondary antibody (antimouse IgG) conjugated to gold nanoparticles and their ICPMS detection after dissolving. The nonspecific adsorptions were found to be around zero. The limit of detection for peptide-modified DNA was 0,2 pmol. The method may have significant potential as an important ICPMS-based nonradioactive DNA detection method for the simultaneous determination of various sequences by labeling different kinds of inorganic nanoparticles.

INTRODUCTION

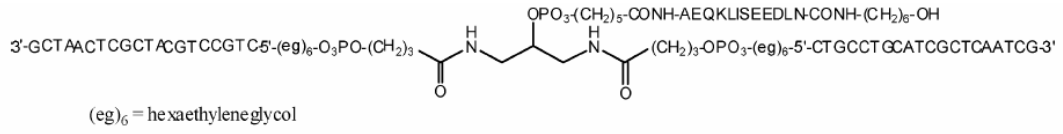
The detection of DNA hybridization is of central importance to the diagnosis and treatment of genetic diseases, for the detection of infectious agents, and for reliable forensic analysis. Recent activity has focused on the development of DNA hybridization assays based on various labeling techniques. To improve the DNA assay sensitivity, to arrive at better and more reliable analysis as well as the simultaneous detection there is a great demand for labels with higher specific activity.

Last, nanoparticles have been using as a novel label for DNA hybridization detection. In general, this novel labeling technology is based on the unique physical properties of metal nanoparticle materials, including large extinction and scattering coefficients, catalytic activity, surface electronics, and efficient Brownian motion in solution (1,2).

Gold nanoparticles that are functionalized with proteins have long been used tools in the biosciences (3). Mirkin et al. have shown how oligonucleotides can be used to modify the surfaces of such particles.4 Interesting applications of nanoparticles for the development of electrochemical DNA sensors are reported (5). Gold nanoparticles are excellent candidates for bioconjugation. They are biocompatible, bind readily to a range of biomolecules such as amino acids (6), proteins/enzymes (7), and DNA (8), and expose large surface areas for immobilization of biomolecules. Gold-based bioassays have been generally based on SPR (9) or electrochemical detection (5,7).

Inductively coupled plasma mass spectrometry (ICPMS) have been already used to study the atomization of nanoparticles for developing a novel nonradioactive immunoassay by coupling this technique with the sandwich-type immunoreaction. For example, the goat anti-rabbit immunoglobulin G (IgG) labeled with colloidal gold nanoparticles has served as analyte in ICPMS for the indirect measurement of rabbit anti-human IgG. A relatively good correlation between the proposed method and enzyme-linked immunosorbent assay has been reported (10). An ICPMS-linked immunoprecipitation assay involving protein A and gold nanoparticlemodified antibodies have also been developed in a similar way (11).

Chart 1. Sequence of the Oligonucleotide-Peptide Used



We report an attempt to measure directly the atomic composition of gold nanoparticles conjugated to oligonucleotides carrying the c-myc peptide via antibody interaction by the use of ICPMS. Oligonucleotide-peptide conjugates are chimeric molecules made by oligonucleotides covalently linked to peptide sequences. They are produced to transfer some of the biological or biophysical properties of peptides to synthetic oligonucleotides (12,13). The introduction of peptides into oligonucleotide sequences has resulted in the introduction of a higher number of multiple nonradioactive labels (14). Recently, the introduction of epitope peptide sequences into oligonucleotides as a nonradioactive labeling system was also described (15).

EXPERIMENTAL SECTION

Apparatus and Procedures. An ICPMS instrument (model PqExCell, Thermo Elemental, Windsford, U.K.) was used for the gold quantification. A UV-visible spectrophotometer (Kontron Uvikon 943) was used for the oligonucleotide quantification. A dot-blot manifold apparatus (Biorad, 162-

0112) attached to a suction device was used for the nonradioactive detection of oligonucleotides carrying the c-myc peptide.

Reagents. Oligonucleotide-peptide conjugate, with a c-myc peptide with a structure as shown in Chart 1, have been prepared as reported previously (16).

Other reagents included mouse monoclonal antibody antic- myc (9E10, hybridoma supernatant), goat anti-mouse-Au colloidal (Sigma, G-7652; see more details in Supporting Information), goat anti-mouse-HRP (Jackson) phosphate buffer saline (PBS: 150 mM NaCl, 20 mM sodium phosphate, pH 7.4), Tween 20 (Merck), 0.5 M NaCl (Merck), and bovine serum albumin (BSA) (Calbiochem).

Nitrocellulose membrane, 0.2 μm, and transfer medium (Bio-Rad, Ref 162-0112) were used for the dot- blot experiment.

The lyophilized oligonucleotides samples were reconstituted with 1 mL of Milli-Q water. The oligonucleotide quantification was carried out at 260 nm by using the UV-visible spectrophotometer, and the concentrations of the oligonucleotides were determined. Stock solutions of 830 μg/mL of the c-myc-oligonucleotide and a 660 μg/mL of the oligonucleotide without peptide were prepared.

All the reagents used for the digestion and preparation of the samples and standard solutions for ICPMS measurements were of spectroscopic grade. Milli-Q water was used throughout the reagent preparation.

Experimental Procedure. Assay Protocol of the Oligonucleotide Carrying the c-myc Peptide. The assay protocol was conducted following the steps of a typical dot-blot assay (Figure 1). Serial halffold dilutions of oligonucleotide starting from 8 μg/ dot were applied onto the nitrocellulose membrane. The peptide-conjugated oligonucleotide is immobilized by UV radiation. UV radiation or baking at 70 ºC are the standard methods to immobilize nucleic acids on nitrocellulose or nylon membranes. They are especially indicated for long genomic DNA sequences. UV cross-linking is preferred for small DNA fragments. Extensive UV cross-linking that may interfere with hybridization (particularly in A-T sequences where T-T dimers can be produced) is avoided by using short-time UV exposures as we do in our experiment (15 s). The membrane was then blocked with PBS containing 1% Tween 20 for 1 h. The membrane was washed for three times with PBS and then incubated at 4 ºC overnight with the primary antibody (9E10 anti-c-myc hybridoma supernatant).

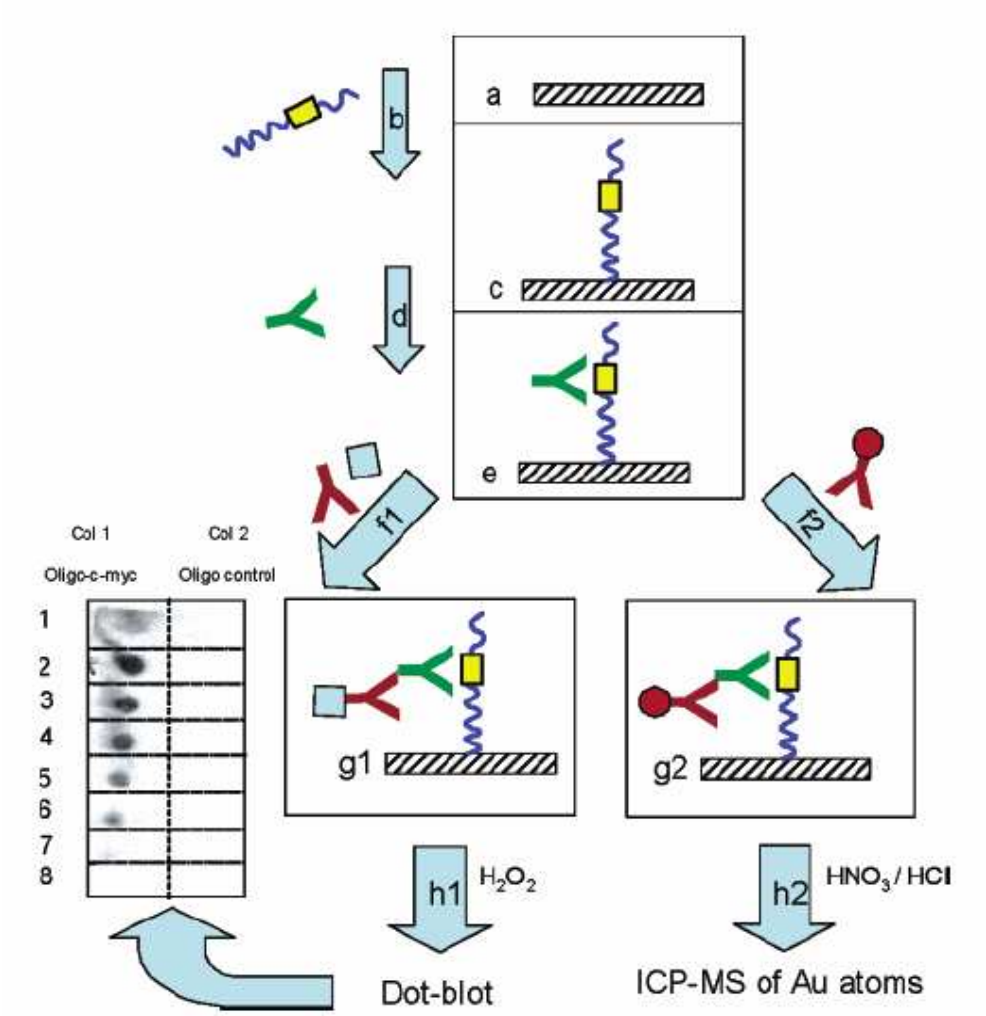


Figure 1. Schematic presentation of the assay protocol. The nitrocellulose membrane (a) was introduced into the dot-blot manifold. The oligonucleotide carrying c-myc peptide (b) is immobilized over the membrane (c). It reacts overnight with the anti-cmyc (d). The immobilized oligonucleotide (e) is then treated: 1. According to the dot-blot assay it reacts first with the anti c-myc and goat antimouse HRP-conjugate antibody (f1) and then developed (h1) following the ECL (Amersham) protocol and exposing to X-ray film. 2. According to the ICPMS-linked assay it reacts for 1 h with goat anti mouse colloidal Au (f2) and then is dissolved (h2) and detected by ICPMS (see details in Supporting Information). Also shown in that figure are the following: autoradiography of the oligonucleotide with peptide c-myc (column 1), and the oligonucleotide without peptide (column 2 - blank). Amounts of oligonucleotide-peptide conjugates: 8 (1), 4 (2), 2 (3), 1 (4), 0.5 (5), 0.25 (6), 0.125 (7), and 0 (8) μg of oligonucleotide/dot.

Table 1. ICPMS Operating Parameter

Generator power	1350 W
Generator frequency	27.15 MHz
Integration time	120 s
Ar plasma flux	13-15 $L \cdot min^{-1}$
Auxiliary Ar flux	0.90-0.95 $L \cdot min^{-1}$
Nebulizer Ar flux	0.85-0.95 $L \cdot min^{-1}$
Peristaltic pump flux	2 $mL \cdot min^{-1}$
Acquisition time	30 s
Washing time	80 s
mode	Peak jump
Channels/mass	100
scans	20
Time/mass	10000 μs

Detection via Dot-Blot Assay. To carry out the detection via enzymatic recognition (see Figure 1, f1-h1), the membrane was incubated with anti c-myc (9E10 hybridoma supernatant) overnight at 4 ºC. The membrane was washed with PBS 1x+ 1% Tween 20 four times for 10 min at room temperature and incubated with goat anti-mouse-HRP conjugated antibody (Jackson) for 1 h at room temperature. The blot was further developed with ECL (Amersham) and exposed to X-ray film.

Detection via ICPMS-Linked Assay. The membrane was incubated with anti-c-myc (9E10 hybridoma supernatant) overnight at 4 ºC. It was then washed with PBS 1x + 1% Tween 20 four times for 10 min at room temperature and finally incubated with goat anti-mouse-colloidal gold conjugated antibody (Sigma G-7652) diluted 1:100 in 0.5 M NaCl buffer containing 0.1% BSA, 0.05% Tween 20, and 5% fetal bovine serum for 1 h at room temperature.

The membrane was cut carefully into small and identical squares according to the deposition areas of DNA. Each square was precisely weighed and introduced into a 2-mL glass vial, where it was digested with 0.5 mL of aqua regia. (Please pay special attention to this hazardous procedure!) After the digestion was completed, each sample was diluted to 10 mL in water and analyzed by ICPMS. The ICPMS operating conditions are described in the Table 1.

To be able to discard unspecific interactions of the different reagents used in ICPMS-linked experiments, three blank assays were designed (see Figure 2, upper part). The first blank was thought to prove the unspecificity of the secondary antibody with the peptide of the oligonucleotide; so a direct incubation of the secondary antibody with the oligonucleotide was done (without the primary antibody incubation). The second blank was thought to prove the unspecificity of the primary antibody with the membrane, after being blocked, so a direct incubation of the primary and the secondary antibodies onto the membrane was carried out (without any oligonucleotide). The third blank was thought to prove the unspecificity of the secondary antibody with the membrane, after being blocked, so a direct incubation of the secondary antibody onto the membrane was performed (without any oligonucleotide nor primary antibody).

All the blanks were treated as explained above and the cut membranes were digested and measured by ICPMS.

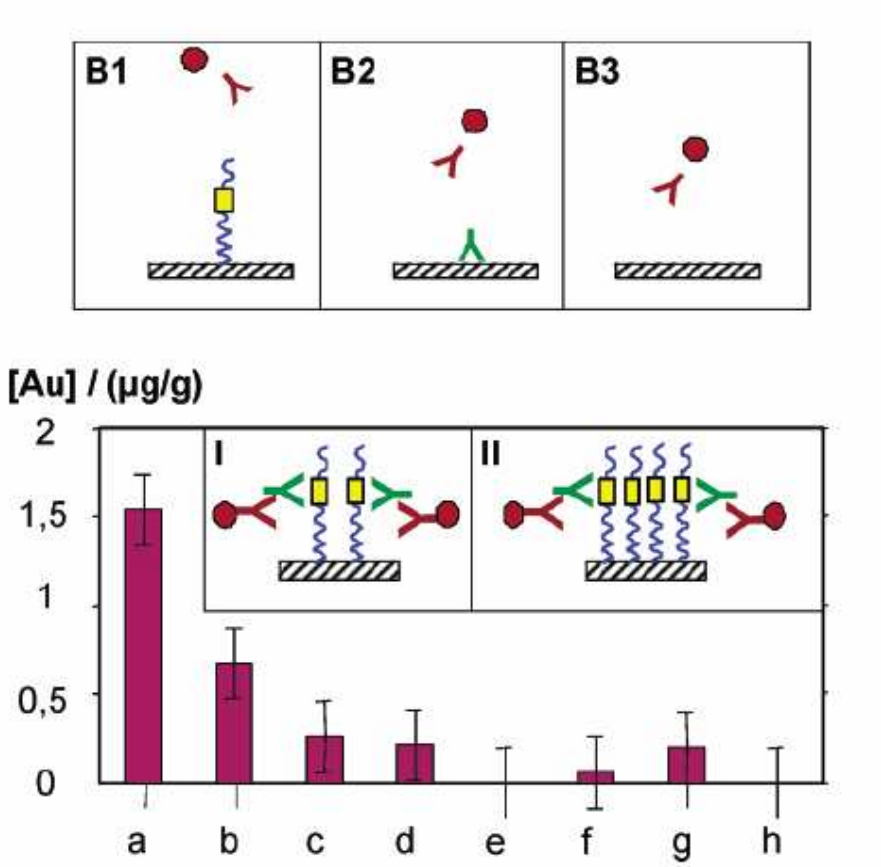


Figure 2. Upper part: schematic of the three blanks (B1, B2, B3) used for the ICPMS-linked assays. Lower part: gold signal obtained for the positive ICPMS-linked assays with decreasing concentrations of the oligonucleotides: 8 (a), 4 (b), 2 (c), 1 (d), 0,5 (e), 0,25 (f), 0,125 (g), and 0 (h) µg of oligonucleotide/dot. Inset figure: schematic representation of phenomenon that explains the inhibitory effect of oligonucleotides.

RESULTS AND DISCUSSION

Two strategies, the classical dot-blot (17) format as reference method and ICPMS, the proposed one, have been used to detect the c-myc peptide epitope attached to synthetic oligonucleotides (see Figure 1). For both cases, oligonucleotide-peptide conjugates were first applied to a nitrocellulose membrane using a manifold attached to a suction device. After immobilization of the oligonucleotide by UV radiation, the samples were incubated with an anti-c-myc monoclonal antibody. In the case of the dot-blot (18) format strategy, it was followed by incubation with a secondary antibody conjugated to horseradish peroxidase and development with luminol as chemiluminescent substrate. The detection limit was 0.125 μg (8 pmol) of oligonucleotide/dot. In the case of ICPMS strategy, it was followed by incubation with the secondary antibody (anti-mouse IgG) conjugated to gold nanoparticles and their ICPMS detection after dissolving.

The enhanced DNA signals obtained by ICPMS of gold tags are combined with the high specificity of oligonucleotide-peptide conjugate interaction with anti-c-myc monoclonal antibody followed by immunoreaction with the secondary antibody (anti-mouse IgG) conjugated to gold nanoparticles.

Since the colloidal Au nanoparticles with extremely high particle density have strong adsorption activity, it is necessary to determine the extent of the nonspecific binding (NSB) of the goat anti-mouse-Au colloidal in the assay. Au signals from NSB were almost zero by adding the secondary antibody and the oligonucleotide but not the primary antibody (B1), the primary and the secondary antibody without any oligonucleotide (B2), or even the secondary antibody only without any oligonucleotide or primary antibody (B3), indicating an excellent NSB level.

The efficiency of the immobilization of c-myc-oligonucleotide is reflected in the obtained results. It depends on the immobilization parameters and directly affects the sensitivity as well as the reproducibility of the method.

Taking in the consideration that the detection limit of the ICPMS is at ~20 ng of Au/g of solution, it can be deduced that 3 ng/dot that corresponds to 0.2 pmol (equal with 200 fmol) of DNA can be detected. This detection limit is ~40 times lower compared to the dot-blot format. By using a gold nanoparticle/ latex microsphere-based colorimetric detection method, Mirkin et al. found that for a single-strand DNA the detection limit was 500 pM (19). The detection limit can be further lowered by using the 1:1 Au nanoparticle-antibody in analogy with a Au nanoparticle- DNA conjugate that avoids the creation of an interconnected network of Au-DNA immobilization platforms (20).

For the studied concentration range of the oligonucleotides (Figure 2, lower part), an inhibitory effect might have occurred and is rather evident at the low-concentration range. This phenomenon could be produced by a steric impediment of the antibody recognition (schematic inset). The use of other oligonucleotides carrying the c-myc peptide at the extreme part should decrease the inhibitory effect observed due to a better exposition during the antibody recognition and consequently increase the sensibility as well as decrease the detection limit. The use of other metal nanoparticles with a higher ICPMS sensitivity will certainly improve the oligonucleotide detection limit.

CONCLUSIONS

This ICPMS-linked DNA detection offers several advantages. The tag is directly analyzed giving the possibility for oligonucleotide quantification at even very low detection limits. It is reasonable to expect that multiple tagged secondary antibodies connected with primary peptide sequence antibodies can be used for simultaneous determination in the same sample. The immediate acidification of the reacted and separated sample allows for long-term storage before analysis and simplifies assay protocols. The stability of nanoparticles under UV and visible light makes possible a long-term monitoring of the environment, for instance, for biological warfare agents or natural pathogenic organisms such as *cholera* and *Escherichia coli*, which represent a critical problem for countries with limited water treatment capabilities.

Although in a very early stage, the proposed ICPMS-linked DNA assay may have significant potential as an important nonradioactive DNA detection method for the simultaneous determination of various sequences by labeling different kinds of inorganic nanoparticles and taking also advantage of the recent development of the ICPMS technique. This study will also be of interest for the development of novel nylon membrane genosensors (21) and DNA chips based on multiple labeling (22) by specific immunoreactions with the peptide sequences introduced into the DNA probes. The possibility of using the large diversity of available antibodies and their many epitope sequences makes oligonucleotide-peptide conjugates good candidates for the directed assembly of complex nanoparticles (16). The DNA modification via peptide method and consecutive antigenin/antibody reaction can be even used for developing multiple genosensor platforms based on the same label. Moreover, this approach can solve the problem of nonspecific adsorption coming from the limited available attachment mechanisms such as biotoin/streptavidin or covalent reactions used to label the DNA strands (23).

The proposed method is a general one with a broad range of application possibilities offered by metallic labels easily detected by the ICPMS technique. The application of this method in real samples is still in process in our laboratories and will be the object of future publications.

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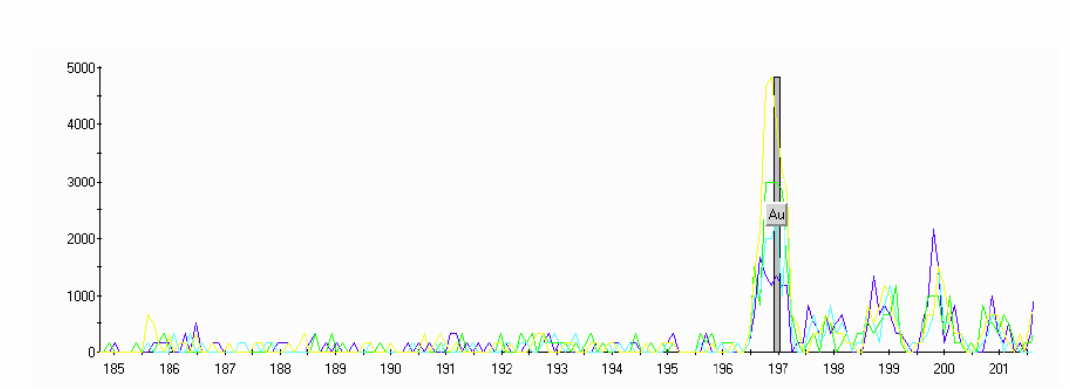
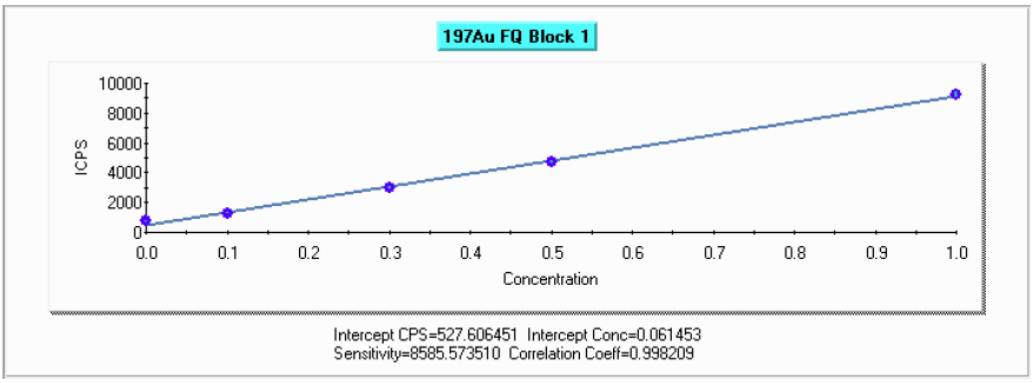
Supporting Information



Supporting Information, Figure S-1
Au ICP-MS Spectra of Au (m/z = 197). Fast scan along the mass range (20 sweeps, 300ms, 10 channels per mass, acquisition time 4s). Lines: Dark blue: 0,1 ppb; green: 0,3ppb; yellow: 0,5ppb; sky blue: sample (Au coming from Au-DNA-membrane digestion) .



Supporting Information, Figure S-2
Au calibration line in peak jump mode. Shown are the ICPMS counts vs. gold concentration in ppb. Conditions: 100 sweeps, 1 channel per mass, 6 s acquisition time.

Data on the Goat anti-Mouse- Au colloidal (Sigma, G-7652) used.
It is an affinity isolated antibody to mouse IgG (whole molecule) 10 nm colloidal gold labeled (monodisperse). According to Sigma information on this product the antibody is conjugated to gold by a modification of Geoghagan's method (1). The supplied conjugate can be tested for immunoreactivity using the dot blot assay of Brada and Roth (2). The product is supplied as suspensions in 20% glycerol with 1% BSA, 0.02 M Tris buffered saline, pH 8.0 and 0.05% sodium azide.
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