Application of 1 nm gold probes on paraffin wax sections for in situ hybridisation histochemistry

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Abstract

An in situ hybridisation technique that uses 1 nm immunogold reagents and silver enhancement was devised to detect biotinylated DNA viral probes in formalin fixed, paraffin wax sections of human cervix. DNA probes labelled with biotin-11-deoxyuridine triphosphate were detected after hybridisation to nucleic acid sequences by an anti-biotin antibody, followed by a gold labelled secondary antibody. Silver enhancement then permitted visualisation of the signal at the light microscopic level. The method was reliable and produced less background staining than previously described methods. The signal could be enhanced by epi polarisation microscopy. Furthermore, biotinylated DNA probes may be detected directly by a 1 nm gold labelled goat anti-biotin antibody without loss of labelling intensity, and this may be preferable to the longer two layer technique, previously described.

We recently described an in situ hybridisation technique using an immunogold silver staining detection system.¹ That technique utilised a 5 nm immunogold reagent visualised at the light microscope level by silver enhancement. The biotinylated DNA probe was detected by a two layer immunohistological technique using a rabbit anti-biotin antibody, followed by a goat anti-rabbit immunoglobulin labelled with 5 nm gold. This complex was then visualised by silver enhancement using a modified physical developing solution.² This paper describes two alternative detection techniques which use 1 nm immunogold reagents.

Methods

Sections (5 µm) of formalin fixed, human cervical tissue showing koilocytosis and cervical intraepithelial neoplasia (CIN) grade II were cut on to single well slides (type PH106 C A Hendley, Loughton, Essex). The slides had previously been coated with 3 amino propyl 1 trioxysilane (Sigma Chemical Co Ltd, Poole, Dorset).³ The slides were placed on a hot plate overnight at 56°C to ensure maximum tissue adhesion. Sections were dewaxed in xylene at 37°C for 30 minutes, at room temperature for 10 minutes, and then washed in absolute alcohol for 2 × 10 minutes. Sections were then hydrated through graded alcohols to distilled water and pro-

cessed according to the method described previously.¹

DNA PROBES

Human papilloma virus (HPV) 6b probe (Professor H Zur Hausen, Heidelberg) was used. Placental human DNA (Oncor Inc, Gaithersburg, USA) was used as a positive control probe for total DNA and plasmid PBR 322 (Gibco) was used as a negative control probe. The probes were biotinylated by nick translation with biotin-11-deoxyuridine triphosphate following standard protocols and applied at a concentration of 200 ng/ml in a hybridisation buffer containing 2 × SSC (sodium chloride, sodium citrate), 5% dextran sulphate, 0.2% milk powder and 50% formamide. (1 × SSC = 0.15M sodium chloride, 0.015M sodium citrate.)

IN SITU HYBRIDISATION

Hybridisation mixture (75 µl) containing the probes was pipetted on to pretreated sections. The sections were covered with a piece of gel bond (ICN Biochemicals, High Wycombe, Buckinghamshire), cut to coverslip size, and used hydrophobic side down. The gel bond was sealed down on to the slides with nail varnish and slides incubated at 90°C for 10 minutes to denature cellular and probe DNA. Hybridisation was carried out at 42°C for 16 hours in a humid chamber. After this the gel bond covering the wells was removed and the slides immersed in 2 × SSC. Sequentially the slides were washed in 2 × SSC at 60°C for 20 minutes, 0.2 × SSC at 42°C (moderate stringency) for 20 minutes, and 0.1 × SSC for 10 minutes before detection of the hybridisation signal. The biotinylated probe was detected by an indirect (two layer) immunocytochemical technique using a rabbit anti-biotin antibody, followed by a goat anti-rabbit antibody adsorbed to colloidal gold or by a direct (one layer) technique using a goat anti-biotin antibody adsorbed to colloidal gold. In both methods the gold labelled antibodies were visualised by silver enhancement.

SIGNAL DETECTION (TWO LAYER TECHNIQUE)

1 Hybridised sections were placed in Lugol's iodine for two minutes, rinsed in water, and decolourised in 2.5% aqueous sodium thiosulphate. Sections were then washed in tap water, followed by TRIS buffered saline (pH 7.6) for five minutes.

2 Sections were treated with rabbit anti-biotin (Enzo Biochem Inc, New York, USA) diluted 1 in 20 in TRIS buffered saline (pH 7.6) for 60 minutes.

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- 3 Sections were washed in TRIS buffered saline (pH 7.6) for five minutes.
- 4 Sections were then treated with a blocking buffer containing 0.8% bovine serum albumin, 0.1% gelatin, 5% normal goat serum, 2 mM sodium azide in TRIS buffered saline (pH 7.6) for five minutes.
- 5 Excess blocking buffer was shaken off and sections were treated with goat anti-rabbit G1 Auroprobe One (Amersham International, Aylesbury, Buckinghamshire), diluted 1 in 100 in a solution of 0.8% bovine serum albumin (BSA), 0.1% gelatin, 1% normal goat serum, 2 mM sodium azide in TRIS buffered saline (pH 7.6) for two hours.
- 6 Sections were washed in TRIS buffered saline (pH 7.6) twice for five minutes, followed by distilled water twice for five minutes.
- 7 Silver enhancement was carried out using a physical developing solution (table). Silver intensification was carried out until sections appeared optimally developed under light microscopic control. Silver enhancement was carried out in a dark room using Ilford safe light S902 or F904.
- 8 Sections were washed in distilled water for five minutes.
- 9 Sections were fixed 2.5% sodium thio-sulphate for three minutes.
- 10 Sections were washed in tap water for one minute.
- 11 Sections were counterstained as desired.
- 12 Sections were dehydrated, cleared, and mounted in synthetic resin.

SIGNAL DETECTION (ONE LAYER TECHNIQUE)

- 1 Hybridised sections were immersed in Lugol's iodine for two minutes, rinsed in water, and decolourised in 2.5% aqueous sodium thiosulphate. Sections were then washed in tap water, followed by TRIS buffered saline (pH 7.6) for five minutes.
- 2 Sections were then treated with blocking buffer as described in step 4 of the two layer technique.
- 3 Excess buffer was shaken off and Auroprobe one, goat anti-biotin (Amersham International) was diluted 1 in 10 in 0.8% bovine serum albumin, 0.1% gelatin, 1% normal goat serum, 2 mM sodium azide in TRIS buffered saline (pH 7.4) for two hours.
- 4 Sections were then treated as described in steps 6 to 13 of the two layer technique.

Results

Both the two step and one step techniques

Physical developing solution

Solution	Reagent	Volume required for 100 ml of developer
1	Gum Acacia 500 g/l	7.5 ml, diluted to 60 ml, in distilled water
2	Citrate buffer, pH 3.5	10 ml
3	Hydroquinone 0.35 g/15 ml	15 ml
4	Silver lactate 0.11 g/15 ml	15 ml

All solutions were prepared in distilled water and mixed in the above order.
Solution 1 was prepared by overnight stirring and filtering through gauze.
Solution 2 was prepared by dissolving 23.5 g trisodium citrate 2H₂O and 25.5 g of citric acid 1H₂O in 100 ml of distilled water.
Solutions 1 and 2 may be kept as stock solutions.
Solutions 3 and 4 were prepared immediately before use.

Figure 1 Cervix showing hybridisation with HPV 6b (arrowed) using the two step method.

Figure 2 Cervix showing hybridisation with HPV 6b (arrowed) using the one step method.

gave reproducible and consistent results with the DNA probes used on formalin fixed, paraffin wax embedded sections of human cervix (figs 1 and 2). The 1 nm immunogold probes gave increased labelling efficiency and less background staining compared with DNA probes, shown by the two layer technique utilising 5 nm immunogold reagents, as described previously.¹

Discussion

Immunogold silver staining produces a signal that is insoluble in common laboratory dehydrating and clearing agents, is visible with low power light microscopy, and which can be enhanced by epi polarisation.¹ Endogenous pigments cannot be confused with the intense reaction product and endogenous enzymes do not interfere with interpretation of results.

Immunoglobulin labelled with 5 nm colloidal gold particles can be regarded as protein coated gold particles. Reduction of the gold marker particle size to about 1 nm results in a reduction of the overall probe size, greater penetrating ability, and less steric hindrance. The smaller the probe size the more silver enhanceable gold nuclei reach the antigenic

site. Immunogold reagents (1 nm) have been manufactured so that the ratio between probe antibody and gold particle is smaller than or equal to 1. Each probe antibody has at least one 1 nm gold particle absorbed to it and may be likened to fluorescence labelled antibodies. The 1 nm gold probes, however, are particulate, not diffuse, and can be readily and precisely localised after silver enhancement. Furthermore, biotinylated DNA probes may be detected directly by a 1 nm gold labelled goat antibiotin antibody as described in the one layer technique, without loss of labelling intensity, and thus may be preferable to the longer, two layer immunohistochemical procedure, described previously.

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- 2 Holgate CS, Jackson P, Pollard K, Lunny D, Bird CC. Effect fixation on T & B lymphocyte surface membrane antigen demonstration in paraffin processed tissue. *J Pathol* 1986; 149:293-300.