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Colloidal Gold Staining and Immunoprobing of Proteins on the Same Nitrocellulose Blot

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Proteins blotted onto nitrocellulose can be stained with colloidal gold. They retain their immunoreactivity so that the stained blot can be probed with appropriate antibodies. The resulting antigen–antibody reaction is made visible by a peroxidase-coupled antispecies antibody. The blue peroxidase reaction product contrasts well with the red-stained protein pattern and allows an easy documentation by black-and-white photography.

KEY WORDS: electrophoresis; protein blot; protein staining; colloidal gold; immunodetection.

Electrophoretic transfer of proteins from polyacrylamide gels onto nitrocellulose (NC)[1] sheets, followed by probing of individual proteins with antibodies (1), became a widespread tool in many fields. Very often, it is desirable to compare the stained pattern of the protein bands with the pattern obtained by the antigen–antibody reaction. To this end, several procedures have been proposed such as preparation of duplicate blots and staining the protein pattern on one blot with, e.g., Coomassie blue, amido black, colloidal gold (2), or India ink (3), and using the second blot for the immune reaction. Procedures using only one blot include staining of the blot with India ink (4) or Coomassie blue (5) before performing the immune reaction with ^{125}I-labeled antibodies, which can then be detected on an autoradiographic film. Alternatively, it is possible to destain the blots during (5) or after (6) the immune reaction and to use enzyme- or gold-labeled antibodies to visualize the immune reaction. Finally, it is proposed (Janssen technical bulletin for "AuroDye") to perform the immune reaction (with gold-coupled secondary antibody followed by silver enhancement) first and to use Tween 20, rather than exogenous protein, for the blocking of unreacted binding sites on the NC. This would allow the staining of the blot afterward with colloidal gold. Among the drawbacks of these procedures is notably an altered immunoreactivity of proteins after Coomassie blue staining (5) or, for the Janssen protocol, an immunoreactive background due to blocking with Tween only and a not easily distinguishable difference in color between the brown immune reaction and the red protein stain. The major problem of the mentioned techniques, except for the Janssen method, is that the protein pattern and the immune reaction cannot be viewed simultaneously on the same blot. We propose here a simple and sensitive method to overcome these disadvantages. We stain the protein pattern first with colloidal gold and, after blocking, perform the indirect immune reaction using peroxidase-labeled antibodies. The red color of the colloidal gold and the blue of the peroxidase reaction product are easily discriminated visually. As it is often necessary to prepare suitable black-and-white photographs, we also

[1] Abbreviations used: NC, nitrocellulose; RAM–peroxidase, rabbit–antimouse antibodies, coupled to peroxidase; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

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discuss appropriate photographic procedures.

MATERIALS AND METHODS

The present method was developed during our work on the function of poliovirus-coded proteins. As a test system, therefore, we used cytoplasmic extracts of polio-infected HEp-2 cells and monoclonal antibodies against the VP0/VP2 capsid (clone 14/1.D1.1) and the 2BC/2C noncapsid polio proteins (clone 11/1.C3) (7). Ten percent SDS–polyacrylamide gels were prepared and loaded as described (8). To test the sensitivity of our method, fourfold serial dilutions of a virus-infected cytoplasmic extract containing polioviral antigens were employed. The dilutions were prepared either in SDS–sample buffer or in uninfected cytoplasmic extract to test the influence of background proteins, which are not immunoreactive, on the intensity of the immune reaction. After electrophoretic separation of the proteins, they were blotted onto NC (Bio-Rad) at 48 V with cooling in a buffer containing 25 mM Tris–192 mM glycine, pH 8.4, 20% methanol, and 0.02% SDS for 2.5 h (1). The blots were then briefly washed in 25 mM Tris, pH 7.4, processed immediately or air dried, and stored at 4°C.

Colloidal gold was either purchased as AuroDye from Janssen Pharmaceutica (Belgium) or prepared by the citrate method (9) by adding 4 ml of 1% Na-citrate to 100 ml of boiling 0.01% chloroauric acid ($HAuCl_4$). Siliconized glassware and double-distilled water was used and all solutions were filtered through 0.45-μm Millex (Millipore) filters. The mixture was boiled for 12 to 15 min so that a bright red color was achieved. This resulted in 20-nm gold grains (measured by electron microscopy) with an OD_{515} of 1 to 1.1 and a pH of 6. Adjusting the pH of the gold solution in the range between 3.7 and 8.0 did not change its staining properties for the blots and so it was used without pH adjustment. Staining of the blots with the self-made colloidal gold ("citrate gold") was done for 30 min to 4 h at room temperature on a shaker. Before and after staining, the blot was rinsed in 0.1 M Tris buffer, pH 7.4.

Immunostaining for the detection of the viral antigens was done after blocking the NC in 0.1 M Tris, pH 7.4, 0.25% gelatin, and 3% ovalbumin (10) by incubating the blots in a 1:10 dilution of hybridoma supernatant overnight at room temperature with constant agitation. Bound monoclonal antibodies were visualized by incubating the blots in a 1:500 dilution of RAM–peroxidase (Nordic, The Netherlands) for 2.5 h at room temperature followed by a further incubation in 0.018% 4-chloro-1-naphthol (Merck, FRG) and 0.006% H_2O_2. After the primary antibody reaction, the blot was washed in 50 mM Tris, pH 7.4, with 150 mM NaCl, 5 mM EDTA, 0.25% gelatin, and 0.5% NP40. After the secondary antibody incubation, the washing buffer consisted of Tris, pH 7.4, supplemented with 450 mM NaCl, 5 mM EDTA, and 0.4% Sarkosyl (10).

Gold staining with AuroDye was done as indicated by the manufacturer. The main difference to the staining with citrate gold is an extensive washing of the blot in PBS with 0.3% Tween 20 before incubation in AuroDye for 4 to 18 h, again at room temperature on a shaker. Afterward, the immune reaction was performed as described above.

For comparison, blots were also stained according to the Janssen protocol with the modification that peroxidase instead of gold-coupled secondary antibody was used; after an extensive Tween 20 wash, the blots were incubated overnight, without any further blocking, in the primary antibody in 0.05% Tween. They were washed again in Tween and incubated for 2.5 h with RAM–peroxidase in 0.05% Tween. After the peroxidase reaction was performed with chloronaphthol as above, the blots were stained with AuroDye for 18 h.

Black-and-white photographs of the blots were taken on Technical Pan film 2415 (Kodak, Rochester, NY) and exposed at an

index of 125/22° without and 75/19° with a yellow filter OG1. Developing took place under constant agitation for 3.5 min in undiluted or in a 1:2 dilution of D19 developer (Kodak).

RESULTS AND DISCUSSION

Blots which are to be stained with gold solutions have to be handled with extreme care, since they are very sensitive to mechanical damage (scratches, impression marks) and, especially when AuroDye is used, to impurities from the transfer and staining solutions. AuroDye has an at least 30-fold higher sensitivity in protein staining than citrate gold (Figs 1a and 1b). An important consequence of this high sensitivity is that the protein load of the gel has to be adjusted accordingly in order to obtain a gold stain of good resolution. It can be estimated as follows: if a strip of the gel, from which the blot is to be made, can be properly stained with Coomassie blue, citrate gold will stain the blot adequately (Fig. 1a) but AuroDye will heavily overstain it (Fig. 1b). If the gel has to be stained with the silver staining (11), AuroDye should be used. AuroDye and the citrate gold also show a slightly different affinity for the individual proteins on a NC blot. The protein pattern, therefore, appears different with the two stains (Figs 1a and 1b). This was also found when other protein dyes such as Coomassie blue or India ink were compared (not shown). It was recently reported (12) that a short treatment of the blot with 1% KOH considerably increases the sensitivity of the immune reaction as well as the intensity of an india ink or AuroDye staining, whereas amido black staining was not affected. In our hands, the intensity of the citrate gold staining is also not altered after KOH treatment.

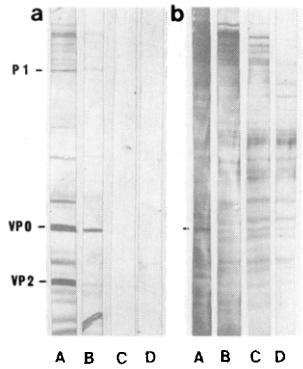

FIG. 1. Comparison of AuroDye and citrate gold staining on Western blots of a poliovirus-infected standard cytoplasmic extract, diluted 1:4 (lanes A), 1:16 (lanes B), 1:64 (lanes C), and 1:256 (lanes D). In (a), the gold staining with citrate gold and the immune reaction are optimal at the same dilutions (1:4), whereas in (b), the gold staining with AuroDye is optimal only at higher dilutions (1:64 to 1:256), where the immune reaction is no longer visible. The monoclonal antibody used recognizes the capsid protein VP2 and its precursors VP0 and P1.

The immunoreactivity of the blots after staining with citrate gold was qualitatively and quantitatively the same as in unstained blots. This was determined by staining one of two identical blots first with colloidal gold before both were subjected to the immune reaction. Figure 2a shows that the viral antigen 2C and its precursors are detected in a particular cytoplasmic extract up to the same dilution (1:16) as in the unstained blot. Diluting the same infected, antigen-containing cytoplasmic extract in an uninfected extract, thus increasing the ratio of nonimmunoreactive protein to antigen (Fig. 2b), does again not change the sensitivity of the immune reaction.

With AuroDye, the same procedure can be performed. The following differences, however, should be observed: the staining with this gold solution has to be prolonged, usually 4 to 18 h, and it results in a slightly bluish color of the blot, which contrasts less well with the peroxidase reaction product than does the bright red of the citrate gold. With AuroDye, the amount of protein in the gel has to be reduced considerably to get individual protein bands stained (Fig. 1b) and, as a consequence, there will also be less antigen present on the final blot. Thus, depending on the composition of the protein mixture stud-

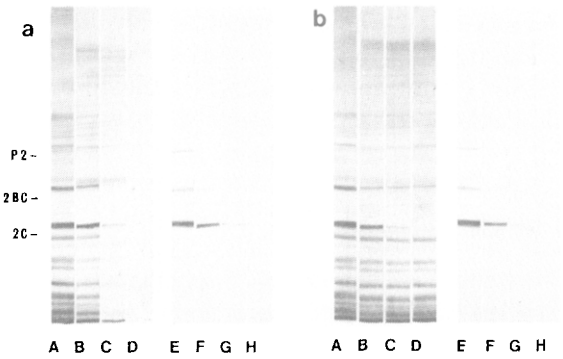

FIG. 2. Poliovirus-infected standard cytoplasmic extract was diluted in sample buffer (a) or in uninfected cytoplasmic extract (b) before it was applied to the gel. The dilutions are lanes A and E, conc.; lanes B and F, 1:4; lanes C and G, 1:16; and lanes D and H, 1:64. No difference in sensitivity of the immune reaction was found whether the blot was stained with citrate gold (lanes A–D) or not stained (lanes E–H) before the immune reaction was performed. Also, the sensitivity of the immune reaction was not impaired by a high load of nonimmunoreactive background proteins (b). The monoclonal antibody recognizes the noncapsid polioprotein 2C and its precursors 2BC and P2.

ied, the antigen may no longer be detectable, which is the case with the viral proteins on our blots (Fig. 1b). In addition, we find that AuroDye slightly lowers the sensitivity of the immune reaction (Fig. 1b).

The methods described above were also compared to two conceptually similar procedures, i.e., the Janssen protocol and the procedure published by Glenney (4). In the Janssen protocol, the immune reaction is performed first, before the blot is stained with AuroDye. The main problems were again arising from the low protein amount that can be processed with the AuroDye which is not sufficient for antigen detection. Substituting gold-coupled secondary antibody followed by silver enhancement (Janssen protocol) for the peroxidase reaction did not change the sensitivity significantly (not shown). In addition, because the AuroDye staining is performed after the immune reaction, Tween 20 is the only blocking agent possible. Our method with the citrate gold stain performed first allows afterward an optimal blocking with exogenous protein (13) and, thus, optimal antigen detection. In the Glenney procedure (4), India ink is used to stain the proteins before the antigens on the blot are visualized by antibodies and ^{125}I-labeled protein A. The sensitivity of the India ink stain is in between that of AuroDye and citrate gold. Due to the dark color of the India ink, however, visualization of the antigen with peroxidase or colloidal gold-coupled antibody results in a very low contrast, so that a radioactive label has to be used which can be detected on an autoradiographic film. Thus, the method yields an antigen detection which is not on the blot itself but only superimposable.

Black-and-white photographs of the final blots processed by our method are best made with a fine-grain pan film such as Kodak Technical Pan 2415, which combines extremely high resolution with sufficient high contrast when developed in D19 developer. In order to have the (blue) immunoreactive bands on the print intensively black and the (red) gold-stained proteins contrastingly gray, it may be necessary to reduce the intensity of the red-colored structures on the film. This can be achieved by using an orange or yellow filter (similar color as the gold stain) which at the same time enhances the contrast of the blue immune reaction (com-

plementary color). Prints can then be made in a conventional way.

In conclusion, the method presented allows us to obtain a clearly stained blot before performing the immunological detection of antigen. Thus, the quality of the blots can be checked before the immune reaction is performed or, in order to save precious reagents or antibodies, only the relevant regions of a blot can be cut out and used for antigen visualization. The method permits an easy localization and photography of the blue-stained antigens within the red protein pattern on the same blot. The relative amount of antigen present in the protein mixture to be analyzed determines the type of colloidal gold to be used for staining the blot; AuroDye can be used when a high amount of antigen is present and, therefore, only a little protein has to be loaded on the gel, whereas citrate gold is used preferably with protein mixtures with a low antigen content, necessitating a relatively higher protein load of the gel.

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