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FerriDye: Colloidal Iron Binding followed by Perls' Reaction for the Staining of Proteins Transferred from Sodium Dodecyl Sulfate Gels to Nitrocellulose and Positively Charged Nylon Membranes

MARC MOEREMANS, MARC DE RAEYMAEKER, GUY DANEELS, AND JAN DE MEY

Laboratory of Biochemical Cytology, Division of Cellular Biology and Chemotherapy, Department of Life Sciences, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse, Belgium

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A staining method for proteins on (positively charged) nylon and nitrocellulose membranes is described. The two-step method uses cationic cacodylate iron colloid which is substituted with Tween 20 at an $OD_{460\ nm}$ = 0.5, followed by Perls' reaction with acid potassium ferrocyanide. It stains transferred proteins deep blue with low background. The sensitivity is intermediate between that of conventional stains and AuroDye, the colloidal gold stain. This is the first sensitive staining method for proteins transferred on (positively charged) nylon membranes. These membranes have documented advantages in immunoblotting. It will therefore be a useful tool for correlating the position of bands or spots of proteins detected with overlay assays with the complete electropherogram in a duplicate protein blot.

KEY WORDS: colloidal iron; protein blotting; protein staining; immunoblotting; electrophoresis; blotting membranes.

During recent years, various techniques for transferring electrophoretically separated proteins to immobilizing membranes have been described (1–4). Such protein transfers are now widely used in overlay techniques to assess binding activities of antibodies, lectins, and various proteins to polypeptide bands [see (5) for a review]. Of the immobilizing membranes, nitrocellulose (NC)[1] paper is the most widely used.

The sensitivity of the overlay techniques on NC paper using various marker systems is very high: subnanogram amounts. This has created the need for "on-blot" protein staining methods which match that sensitivity. Sensitive protein staining methods should be useful tools for controlling the transfer and for making the correct correlation between the transferred electropherogram and bands or spots (two-dimensional gels) detected in the overlay assay. Recently, we have introduced such a protein staining method, which is based on the selective binding of stabilized anionic colloidal gold or silver particles to the proteins and matches this high sensitivity (6).

Positively charged nylon membranes (Zetabind, Zetaprobe, Nytran) reportedly have a considerably higher and more tenacious binding capacity for proteins transferred from sodium dodecyl sulfate (SDS)–polyacrylamide gels than does nitrocellulose (7). This has resulted in even more sensitive overlay techniques. A major drawback of such membranes is that protein transfers can not be easily stained because most protein dyes are anionic, resulting in extremely high background. This is also the case for the colloidal gold or silver stains which are restricted to NC membranes (6).

In this paper, we describe an "on-blot" staining method for proteins transferred from SDS gels to positively charged nylon membranes that is both sensitive and simple. It is

[1] Abbreviations used: NC, nitrocellulose; SDS, sodium dodecyl sulfate; FeC, iron colloid; cac, cationic cacodylate; HMW, high molecular weight; PAGE, polyacrylamide gel electrophoresis.

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based on the selective binding of a cationic cacodylate iron colloid, followed by Perls' reaction (8) with acid potassium ferrocyanide. This results in blue-stained protein bands with only moderate background staining. The method is limited to transfers of SDS gel-separated proteins but also works on unmodified nylon membranes [e.g., Gene Screen (NEN)] and on NC paper with a sensitivity on NC paper which is about eight times less than that of AuroDye, the colloidal gold stain.

MATERIALS AND METHODS

The preparation of Tween 20 cationic cacodylate iron colloid (FerriDye). Cationic cacodylate iron colloid (cac iron colloid) was prepared according to a modification of the method of Seno (9,10). Briefly, 10 ml of 0.5 M $FeCl_3 \cdot 6H_2O$ (Merck) was poured into 60 ml of boiling distilled water (FeC). The ferric chloride solution was added drop by drop under stir to keep the water boiling during the process. The FeC was cooled to room temperature. One volume of FeC was added to 9 vol of 0.1 M sodium cacodylate buffer, pH 7.0, and the optical density at 460 nm was measured. Sodium cacodylate is toxic; therefore it is advisable to wear gloves during all the manipulations. This stock solution was further diluted with sodium cacodylate (BDH) buffer to the working concentration (see below). Tween 20 (Bio-Rad) was added to this diluted cacodylate iron colloid from a 10% stock in H_2O to a final concentration of 0.2%. This Tween 20 cationic cacodylate iron colloid will from now on be called FerriDye in analogy with AuroDye the colloidal gold stain for protein blots on nitrocellulose membranes.

The staining procedure. The basic procedure consists of washing protein blots with distilled water, 3 × 10 min, 200 ml in a large petri dish, and contacting them with FerriDye. Colloidal iron particles in cacodylate buffer are relatively stable at neutral pH and remain cationic (10). We have found that they bind to the SDS-containing proteins but not to positively charged or unmodified nylon membranes nor to nitrocellulose paper.

Initial attempts had shown that FerriDye stained the proteins yellowish brown, with no or very light background. It was known that cac iron colloidal particles give a positive Perls' reaction resulting in Prussian blue (8,10). Prussian blue development of the FerriDye-stained blots resulted in dark-blue protein bands with high contrast and low background.

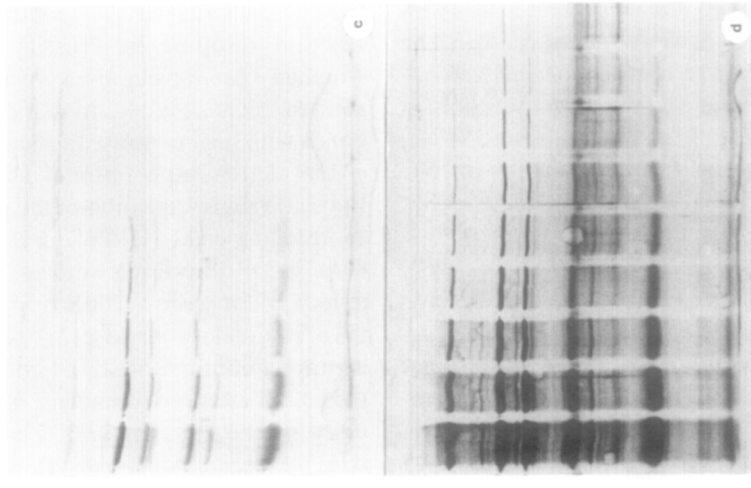
For determining optimal staining conditions and standardization of the method, high-molecular-weight (HMW) protein standards (Lot 24348, Bio-Rad) were used. They consisted of myosin (200K), β-galactosidase (116.5K), phosphorylase *b* (92.5K), bovine serum albumin (66.2K), and ovalbumin (45K), all at approximately 2 mg/ml as indicated by the manufacturer. The HMW standard was separated by SDS–gel electrophoresis according to Laemmli (11) on a 7.5% acrylamide gel. HMW standard stock solution (8.1 μl) was diluted in 400 μl sample buffer, boiled for 3 min, and run in a one-well gel. The separated proteins were electrotransferred to Zetaprobe (Bio-Rad) in 25 mM Tris, 192 mM glycine (Bio-Rad) buffer without methanol, pH 8.30, using an LKB Transphor apparatus.

The blot was briefly washed with water (3 × 10 min, 200 ml) and cut into 3-mm-wide strips. They were then incubated in duplicate with 5 ml of FerriDye, from $OD_{460\ nm}$ 1.5 to 0.05 in a stoppered glass tube for 1 h at room temperature.

On one series of strips, the Perls' reaction was performed. Fresh developer was prepared in a fume hood by mixing 2 vol of water, 1 vol of 0.05 M K_4 $Fe(CN)_6$ (Janssen Chimica) in water, and 2 vol of 1 N HCl.

The strips were thoroughly washed with water (3 × 2 min), reacted for about 1 min in a fume hood, and immediately washed in a large excess of water. The reaction stopped after a while during the washing. The preparation of the developer and the reaction itself must be carried out in a fume hood because acid potassium ferrocyanide may release poisonous hydrogen cyanide gas.

To test the sensitivity of the standard procedure (see below) five 0.75-mm-thick 10-well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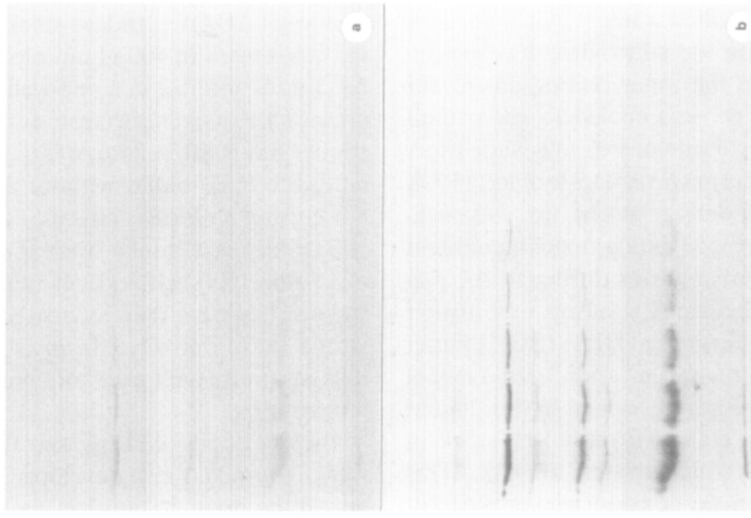
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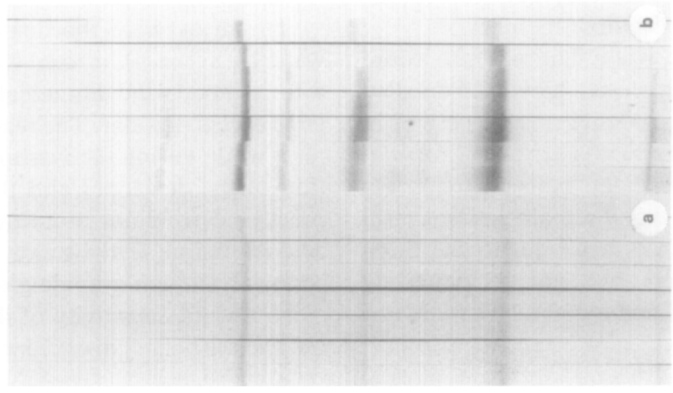
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gels were loaded with a serial dilution series of the HMW standards (1000–3.5 ng per major polypeptide band). One gel was stained with silver (12). Two electropherogram were electrotransferred to Zetaprobe membranes as described above. The two remaining ones were electrotransferred onto nitrocellulose paper (Bio-Rad) using blotting buffer supplemented with 20% methanol (1,6). One Zetaprobe blot was stained with FerriDye at an $OD_{460\ mm} = 0.4$. The other Zetaprobe blot was stained in the same way and treated with the Perls' reaction. One nitrocellulose blot was stained with AuroDye (6)(obtained from Janssen Life Sciences Products) and the other with FerriDye ($OD_{460\ nm} = 0.4$) and the Perls' reaction. The method was also tested on a blot of a whole cell extract of chicken lung epithelial cells prepared as described by Moeremans *et al.* (6) and separated on a 7.5% gel. Pictures were taken on Kodak Plus-X Pan professional B & W film, developed in Kodak D-76, 1:1. 9 min. Printing was done to reflect the real differences in contrast and staining intensity.

For clarity, we outline here the optimal staining conditions for protein blots of SDS–polyacrylamide gel-separated polypeptide bands:

1. After transfer to any type of blotting membrane, the blot is washed in distilled H_2O for 3×10 min.

2. The washed blot is incubated in FerriDye prepared from iron colloid to an $OD_{460\ mm} = 0.5$ as described earlier. The colloid contains sodium cacodylate which is toxic. Therefore it is advisable to wear gloves during the manipulation.

3. Incubation should last 1 h. At the end of the incubation, the major bands are stained yellowish brown.

4. The blot is rinsed in H_2O for 3×2 min.

5. The blot is reacted with developer (acid potassium ferrocyanide) freshly prepared as follows (for 100 ml, add the components in this order):

40 ml HCl 1 N
40 ml H_2O
20 ml $K_4Fe(CN)_6$ 0.05 M

After 1 min the reaction is stopped by washing the blot extensively in distilled water. The reaction continues until it reaches an endpoint. The preparation of the developer and the reaction itself must be carried out in a fume hood.

6. The blot is dried and photographed.

RESULTS

When washed strips of protein blots were incubated with a concentration series of FerriDye from $OD_{460\ mm} = 1.5$ to 0.05 the polypeptide bands gradually stained yellowish brown. It took an incubation time of 1 h to fully develop the contrast. Figure 1a shows the decrease in staining of the bands and background with decreasing FerriDye concentration. The Prussian blue reaction performed on the duplicate series resulted in deep blue-stained protein bands, with formation of a variable background. The best signal to back-

FIG. 1. Determination of the optimal working dilution of FerriDye. HMW standards were run in a one-well gel as described under Materials and Methods and transferred to a Zetaprobe sheet. The washed blot was cut into 3-mm-wide strips and incubated in duplicate with FerriDye at the following optical densities at 460 nm: 1, 1.48; 2, 1.05; 3, 0.85; 4, 0.64; 5, 0.41; 6, 0.20; 7, 0.10; 8, 0.05. (a) The reaction before Perls' reaction; (b) the same series of strips after Perls' reaction. Optimal signal-to-noise ratio was obtained with a FerriDye concentrated at $OD_{460\ nm} = 0.4$ to 0.6.

FIG. 2. Comparison of FerriDye with currently used staining methods. Nine dilutions of HMW protein separated standards (1000–3.5 ng per polypeptide band) were separated on four 7.5% gels. Two of them were transferred to Zetaprobe, the other two on nitrocellulose. (a) FerriDye ($OD_{460\ nm} = 0.4$) without Perls' reaction on Zetaprobe. (b) A duplicate after Perls' reaction. The proteins are stained in deep blue. (c) FerriDye with Perls' reaction on nitrocellulose. The same sensitivity as in (b) is obtained. (d) AuroDye on a duplicate of (c). FerriDye + Perls' is approx eight times less sensitive than AuroDye.

FIG. 3. FerriDye followed by Perls' reaction of an SDS–PAGE-separated complete cell extract, electrotransferred to a Zetaprobe membrane.

ground level was obtained with FerriDye at $OD_{460\,mm}$ = 0.4 to 0.6 (Fig. 1b). The sensitivity of this method as judged from the reaction with serially diluted standard proteins was estimated as 1 ng/mm^2 (Fig. 2b). As shown in Fig. 2c it also functions very well on nitrocellulose membranes with a sensitivity approx eight times less than that of AuroDye (Fig. 2d) [see Ref. (6) for comparisons of sensitivity between AuroDye and conventional staining methods]. Figure 3 shows the staining of a total cell extract. Many of the closely packed bands are highly contrasted.

DISCUSSION

Our results show that cationic cacodylate iron colloid (9,10) supplemented with Tween 20 (FerriDye) can be used to stain protein blots on positively charged nylon membranes and nitrocellulose paper. Results not presented here have shown that this is also the case for unmodified nylon membranes. It was found that optimal results were obtained after development with the Perls' reaction. This reaction is used in histochemistry to visualize colloidal iron bound to anionic sites in tissues [e.g. (10)]. Protein bands become visible in good contrast as deep-blue bands and background is low. The most important factors were the use of 0.1 M cacodylate buffer, pH 7.0, to dilute the iron colloid to its optimal working concentration and addition of Tween 20 to 0.2%. The sensitivity of the method is satisfactory although less than silver staining of the polyacrylamide gel and AuroDye staining of nitrocellulose protein blots.

FerriDye can only be used on SDS-denatured proteins. Results not presented here have shown that it does not stain protein blots of non-SDS–PAGE gels.

The colloidal iron stain for protein blots on any type of blotting membrane is simple and another example of how techniques used in histochemistry can be adapted to blotting techniques. In analogy with AuroDye, we call the stain FerriDye. FerriDye is commercially available from Janssen Life Sciences Products (Beerse, Belgium). It is the first staining method that gives satisfactory results on nylon membranes, which show certain documented advantages. It will therefore be a useful tool for assessing the quality of protein transfer and correlating on such membranes the position of bands or spots of proteins detected with overlay assays with the complete electropherogram in a duplicate blot.

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