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which the DNA runs backwards, but the increase is not linear, which should result in an increase in the thickness of the converged band. In our experience, this is not significant compared to the increased thickness due to diffusion, so we do not bother to adjust the voltage to maintain precisely the same voltage drop per centimeter of gel. If the gel is run backwards at a significantly different voltage (e.g., three times as high), then the DNA does not converge into a narrow band. Even with the same total voltage, the time required for the DNA to converge in the backwards run is noticeably shorter than the time needed to run it forward because of the slightly shorter distance run in reverse and because the voltage drop per centimeter of gel is slightly higher. The DNA converges effectively even if as much as two-thirds of the gel is removed, using the same total voltage in reverse.

The rate of DNA migration is strongly affected by the amount of ethidium bromide intercalated within the DNA molecules (1). Ethidium intercalation partially neutralizes the negative charge of the DNA molecule and also increases its length. If ethidium is not present in the gel, the leading DNA molecules gradually lose ethidium and accelerate their motion through the gel. We put sufficient ethidium bromide in the loading dye to saturate the DNA [which can intercalate one ethidium molecule per 2 bp (1)] and put smaller amounts of ethidium in the gel and in the running buffer (both at 400 ng/mL) so the DNA remains saturated during the run.

We have found this method especially useful in 5' rapid amplification of cDNA ends (RACE) to clone apparent full-length transcripts, in spite of significant degradation of the source RNA and a possible strong stop that limits the size of first-strand cDNA synthesis products. Figure 1 shows three images of the same gel with the same 5' RACE PCR products (Life Technologies, Rockville, MD, USA) in lanes 2 and 3 and 100-bp size standards in lanes 1 and 4. The top image shows the 5' RACE DNA fully run out. There is a long smear with a partly obscured band at 0.6 kbp. Our early cloning efforts had yielded only short partial transcripts of about 0.6 kbp. The middle image shows the gel cut to remove

fragments smaller than 0.7 kbp. The wells are positioned between the white arrows. The lower image shows the gel after running it backwards, with converged DNA bands located close to the wells. TOPO TA cloning (Invitrogen, Carlsbad, CA, USA) of the purified, converged DNA yielded a variety of clones longer than 0.7 kbp, including apparent full-length clones of 1.9 kbp.

While we typically select DNA larger than a specific size, the method can also be used to obtain DNA between a maximum and a minimum size. The gel is cut perpendicular to the migration direction at positions corresponding to the maximum and minimum sizes of DNA desired. The portion of the gel with smaller fragments is discarded. The gel fragment containing the desired sizes is displaced sideways and held against the part of the gel containing the wells so that the desired DNA moves backwards into an otherwise empty lane. The DNA converges close to the well, as usual. We have found that gels may differ significantly in DNA mobility, even gels that are nominally the same; therefore, we recommend using a portion of the original gel for the backwards migration.

We find this method to be very simple and convenient. In addition to 5' RACE, we also use it to size-select mechanically sheared DNA for subcloning. For highly efficient cloning (as in library construction), it is helpful to minimize UV exposure of the DNA; this can be achieved by covering the part of the gel containing DNA for cloning while the standards are being observed. The desired band is cut when the standard bands converge near the wells.

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We thank three anonymous reviewers for their comments. This was supported by National Science Foundation grant no. DEB-9974374 to M.W.F. Address correspondence to Michael W. Frohlich, Herbarium, University of Michigan, 1205 North University, Ann Arbor, MI 48109-1057, USA. e-mail: mtfroh@umich.edu

Received 15 May 2000; accepted 7 November 2000.

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Method for the Immunological Detection of Silver-Stained Proteins on Nitrocellulose Membranes

BioTechniques 30:266-272 (February 2001)

The combination of western blotting and high-resolution 2-D gel electrophoresis represents a rapid and simple method toward the identification of protein spots in complex mixtures such as lysates from organs, tissues, cells, and body fluids. To obtain a reference 2-D protein spot pattern, it is common to stain all proteins in a gel or on a membrane with silver, taking advantage of the high sensitivity of this dye (1,4,5). Unfortunately, the unequivocal assignment of an immuno-stained spot to the corresponding silver-stained counterpart in 2-D gels is difficult because a method to destain silver-stained proteins on a membrane, and thereby allowing the successive detection by immuno-staining, has not been described. To circumvent this problem, protein mixtures are usually separated in parallel on two 2-D gels that are then blotted. While one of the membranes is subjected to immunological detection, the other membrane is stained with silver (8). However, individual handling can lead to regional shrinkage or expansion of the two gels, and as a result, the position of the corresponding protein spots varies to a certain extent (5). This impedes the assignment of an immuno-stained spot to the corresponding silver-stained counterpart. Another method of solving this problem is to visualize the complex protein spot pattern on a 2-D blot with dyes, such

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as amido black or Ponceau S, which are easily removed from the proteins by one washing (6,10). Following documentation and destaining, blots are exposed to specific antisera. Likewise, blots are counterstained with Coomassie® Blue following immuno-staining (12). Because staining with antisera and dyes is performed on the same membrane in these cases, an unequivocal assignment of the immuno-detected spot to a dye-stained counterpart is possible. However, these methods suffer from the low sensitivity of the employed dyes in detecting protein spots (10).

In this report, a combination of the sensitive silver-staining method, destaining, and immuno-detection of proteins that are immobilized on nitrocellulose membranes following the transfer from 2-D gels is described. This new method allows, at high sensitivity, an unequivocal assignment of immuno-stained spots to the silver-stained pattern on the same membrane.

Human embryonic kidney (HEK) 293 or HepG2 cells were grown at 5% CO₂, 37°C, using minimal essential medium with Earl's salts or RPMI 1640 medium containing 5% fetal calf serum, 5% newborn calf serum, and 2 mM L-glutamin. Nuclear extracts were prepared according to Gorski et al. (3). High-resolution gel electrophoresis was carried out according to Görg et al. (2). Forty-two microliters of 1.4 M dithiothreitol (DTT), 4 µL Pharmalyte™ 3-10 (Amersham Pharmacia Biotech, Freiburg, Germany), 4 mg CHAPS, and 108 mg urea were added to 100 µL nuclear extract (about 400 µg protein) and incubated for 1 h at room temperature with shaking. Then, Immobiline™ stripes (11 cm, pH 3-10) (Amersham Pharmacia Biotech) were re-hydrated in this solution, and the sample was submitted to separation in the first dimension. Electrophoresis in 2-D was carried out at 20 mA for 4 h using a 15% SDS-polyacrylamide gel (13.5 cm × 20 cm × 0.75 mm).

Proteins were transferred from the gels to nitrocellulose membranes (Optitrans® BA-S 83; Schleicher & Schuell, Dassel, Germany) using a semi-dry blotter for 1 h, using 2 mA/cm², 25 mM Tris-HCl (pH 8.3), 150 mM glycine, 10% (v/v) methanol (Sammy Dry®; Schleicher & Schuell). The membrane was silver-stained in 100 mL solution

consisting of 75 mL colloid silver (see below), 5 mL Tween® 20, 20 mL 50 mM sodium citrate, pH 3.0, overnight at room temperature (5). Colloid silver was generated by mixing 3.5 mL 40% (w/v) sodium citrate, 2.5 mL 30% (w/v) iron (II) sulfate, and 2.5 mL 10% (w/v) silver nitrate. Silver particles were collected by centrifugation (90 Ti rotor, 57 000 rpm at 20°C for 30 min), suspended in 5 mL water, and diluted with 5 mL 40% sodium citrate. This procedure was repeated three times. Finally, silver particles were suspended in water so that a 1:100 dilution showed an A₃₉₄ of 1. Following documentation by scanning, we incubated the blot in destaining solution [1 volume of solution containing 37 g/L NaCl and 37 g/L CuSO₄, to which NH₄OH was added until all precipitated material re-dissolved completely, 1 volume 436 g/L Na₂S₂O₃, and 2 volumes water (7) until no silver-stain-

ing could be detected (1-5 min)]. The membrane was washed twice with water and once with PBS containing 0.3% Tween 20. For dot blot and western blot analyses, membranes were incubated in PBS containing 3% milk powder and 0.3% Tween 20 to prevent nonspecific binding of antisera and exposure to the primary antibodies (diluted 1:6000) overnight at room temperature. c-jun and c-fos antisera were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA); the anti-Ran-GTPase antiserum was provided by Transduction Laboratories (Lexington, KY, USA). Then, nitrocellulose filters were washed three times in PBS containing 3% milk powder and 0.3% Tween 20 and incubated with secondary antibody conjugated to alkaline phosphatase (1:20 000) for 2 h at room temperature. After the removal of the secondary antibody by three washes in detection buffer

Figure 1. Immunological detection of c-jun (A) and c-fos (B) peptides on dot blots following staining with silver or amido black. Various dilutions (0.5 µL) of the antigenic c-jun and c-fos peptides (Santa Cruz Biotechnologies), dissolved in PBS containing 20 ng/µL BSA and, for negative and positive control, PBS and 100 ng BSA were spotted according to the scheme onto nitrocellulose membranes. One membrane was stained with amido black (A and B, panels I), another with silver (A and B, panels II), and the third remained unstained. Following the destaining of the two membranes, all three membranes were stained immunologically using antibodies raised against the c-jun (A, panels III, IV, and V) or c-fos antigen (B, panels III, IV, and V). Panel V represents the membrane not previously stained with dyes.

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(100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM MgCl₂), we incubated the blot with the substrate 5-bromo-4-chloro-3-indolyl phosphate in the presence of nitro blue tetrazolium in detection buffer, as suggested by the supplier (Sigma, Steinheim, Germany).

For peptide mapping by mass spectrometry, protein spots were excised and submitted to tryptic digestion (7). Peptide masses were analyzed by a matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometer (Bruker-Franzen, Bremen, Germany). Database searches based on peptide maps were performed using the Peptidsearch program (<http://www.mann.embl-heidelberg.de>) with an accuracy of less than 0.5 Da.

To develop a method that allows western blot analysis and high-sensitivity staining of proteins on the same membrane, we adapted an oxidation procedure of elemental silver. This method was used in photo shops to lighten photographs, and it has been used successfully to destain silver-stained gels (4). In dot blot experiments with two different peptides derived from the two transcription factors, c-jun and c-fos (Figure 1), it is shown that this protocol can be employed to destain silver-stained proteins on blotting membranes, allowing for their subsequent

immuno-detection. As expected, silver-stained proteins are detected at about 10- to 100-fold higher sensitivity than proteins stained with amido black (Figure 1, A and B, panels I and II). It is also evident that the silver-staining/destaining protocol did not reduce the sensitivity of the immuno-detection. The immuno-detected spots appear to be of comparable intensity, whether they were previously stained with amido black, or with silver and then destained (Figure 1, A and B, panels III and IV), or not stained at all (Figure 1, A and B, panel V). Both antisera used in these experiments led to visible signals at 0.1–0.5 ng antigenic peptide per spot.

To demonstrate how this protocol can be successfully employed for western blot analyses of complex protein mixtures, a nuclear extract of HEK 293 cells was submitted to 2-D gel electrophoresis. Following electrophoresis, the proteins were transferred from the gel to a nitrocellulose membrane and stained with amido black (Figure 2A). Then, the membrane was destained and silver-stained (Figure 2B). It is apparent that many more spots are visible using the silver-staining protocol (compare Figure 2A and 2B). The silver-stained proteins were then destained using our new protocol before western blot analysis with a monoclonal anti-Ran-

GTPase-antibody. A single protein spot was detected by the antibody-identifying Ran-GTPase (Figure 2C, arrowhead). The Ran-GTPase-specific spot is not visible on the amido black-stained membrane (Figure 2A, arrowhead). It is, however, present on the silver-stained membrane (Figure 2B, arrowhead). The identity of the silver-stained protein with Ran-GTPase was finally proven by peptide mapping and mass spectrometry. The masses of the seven peptides (1244.6, 1689.8, 1784.9, 1971.1, 2052.1, 2180.2, and 2251.1 Da) generated by tryptic digestion of the silver-stained protein spot corresponded to predicted tryptic peptides of the Ran-GTPase protein. Another example showed the general applicability of the method. Nuclear proteins from a different cell line, HepG2 cells, were separated by 2-D gel electrophoresis, blotted, and silver-stained (Figure 2D). Following the destaining of the membrane, western blot analysis was performed with an anti-c-jun-antibody. Two proteins were detected (Figure 2E, arrows) that are very close to, but distinct from, any of the silver-stained spots (Figure 2F, overlay of D and E, circles). Mass spectrometry of all of the proteins in these regions verified that the adjacent silver-stained proteins do not correspond to c-jun. First, this observation underscores that silver-staining/destaining does not significantly reduce the sensitivity of an immuno-detection procedure that allows the identification of proteins present in too low amounts in complex mixtures to be detected by silver-staining. Second, the new method is superior to the conventional technique relying on two parallel experiments, one for immuno-staining and one for silver-staining, which could, in view of the drawbacks mentioned above, result in a false assignment of such narrow spots.

Therefore, our results show that the new protocol allows, at high sensitivity, the unequivocal assignment of immuno-stained spots to the silver-stained pattern. We think that this method is particularly appropriate for the identification of proteins in complex mixtures in regions of high spot densities (i.e., poorly resolved proteins of high molecular weight or where other techniques such as peptide fingerprinting cannot be employed). Therefore, it may be useful for

Figure 2. Western blot analyses of 2-D gels following silver-staining/destaining of the immobilized proteins. Nuclear extracts obtained from HEK 293 cells (A–C) or HepG2 cells (D–F) were separated by 2-D and blotted onto nitrocellulose membranes. The blot containing the HEK 293 cell nuclear proteins was first stained with amido black (A), was destained, and was then stained with silver (B). Following the removal of the silver particles, the membrane was incubated with the anti-Ran-GTPase and a secondary antibody conjugated with alkaline phosphatase (C). Arrowheads point to the position of the Ran-GTPase-spot in the three images. The blot containing the HepG2 nuclear proteins (for clarity, only part of the blot is shown) was stained with silver (D), was destained, and was incubated with the anti-c-Jun-antibody and a secondary antibody conjugated with alkaline phosphatase (E). Panel F shows the overlay of the images shown in panels D and E. Arrows point to the spots detected with the anti-c-jun-antiserum. Circles mark the positions of the spots obtained after immunological detection in the overlay. Please note that the c-jun-spots are not visualized by silver staining.

proteomics that resolve thousands of proteins on 2-D gels that need to be identified. Western blotting remains an important method in proteomics to identify specific proteins in complex protein mixtures (9,11). The staining/densitometry procedure described here will increase the reliability of assigning immuno-stained protein spots to their silver-stained counterparts in the reference 2-D gel pattern.

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Received 26 June 2000; accepted 27 October 2000.

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DNA Isolation from Chloroform/Methanol-Treated Mycobacterial Cells without Lysozyme and Proteinase K

BioTechniques 30:272-276 (February 2001)

The recent resurgence of pathogenic mycobacteria including *Mycobacterium tuberculosis* (9) and *M. ulcerans* (5) increases the relevance of molecular genetic research on the elucidation of pathogenicity and the development of new prophylactic and therapeutic tools against mycobacterial diseases (3). However, the isolation of genomic DNA remains a laborious task despite the availability of several protocols (10) that are generally divided into two groups according to the procedure of cell wall lysis. The group of mechanical cell wall disruption includes short protocols that are often associated with DNA shearing, whereas the group of enzymatic cell wall lysis includes methods that require several hours or days to prepare genomic DNA. In addition, a few methods, based on organic solvent pretreatment (OSP) of cells before the DNA isolation procedure, have been reported for preparation of high molecular weight DNA from mycobacteria (4,6). Although these OSP methods apparently support the preservation of DNA integrity, they are time consuming and labor intensive, as they include refluxing and washing steps to remove traces of organic solvents before enzymatic lysozyme/proteinase K lysis steps. We have developed from existing methods (4,6) a new OSP method that is based on the guanidine thiocyanate disruption (1) of mycobacterial cells pretreated with chloroform/methanol (2:1, v/v). Modifications involve not only the omission of the enzymatic cell lysis including the washing/refluxing steps preceding lysing steps (4,6) but also the use of vortex mixing instead of a Teflon® homogenizer (6) and platform rocker (4). Our method is faster and easier to apply, requiring less than 1 h to purify high molecular weight DNA from mycobacteria, compared with the 6 and 20 h required by