0273-2289/93/4202-03—0181$03.00

Protocols in Biotechnology

Silver Staining of DNA in Polyacrylamide Gels

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ABSTRACT

Nucleic acids can be detected at the picogram level using a quick and simple silver staining method (*1*). Using very thin polyester-backed polyacrylamide gels, a further simplified protocol was compared to other widely used silver staining procedures. The improved protocol described here was the most sensitive, the fastest to perform, and had relatively few steps and reagents. This method also produced the least number of staining artifacts and offered images of high contrast.

Index Entries: DNA; polyacrylamide gels; silver staining.

INTRODUCTION

Short of radioisotopic or fluorescent labeling, detection of trace amounts of biological molecules is best achieved by silver staining. Silver staining can be readily applied to the visualization of nucleic acids with very high sensitivity and has numerous applications, including its use in DNA fingerprinting (*2–4*). The various silver staining methods currently used fall into two categories based on the chemical state of the silver ions that prime the staining reaction (*5*). Alkaline methods use a diamine complex of silver nitrate in a highly alkaline environment and usually develop the image in dilute acid solutions of formaldehyde. In contrast, acidic methods use

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silver nitrate solutions for gel impregnation and usually use alkaline developing solutions containing formaldehyde. Reportedly, alkaline methods are less sensitive, but better suited for thicker gels, whereas acidic methods are rapid, but work best with thin gels (*6*).

We recently reported a simple, fast, and highly sensitive acidic DNA silver stain (*1*) based on a photochemically derived staining procedure (*7*) that allows detection of DNA down to 1 pg/mm^2 band cross-section, avoids background staining of the gel without loss of contrast, has few steps, and takes less time than other published protocols. This method is particularly suitable for staining DNA under more demanding circumstances, such as for the analysis of complex DNA amplification fingerprinting profiles (*4*) or when polyacrylamide gels are polymerized onto polyester backing films. Backed gels have the advantage of easy handling and allow preservation as a permanent record, but are especially difficult to stain with silver, since the backing film serves as a surface for silver deposition and restricts diffusion of reagents in and out of the gel to one face only. Furthermore, we used very thin gels that can assay minute amounts of complex nucleic acids mixtures. This requires utmost sensitivity from the silver stain used. Our silver staining method is versatile, requires minimal handling, has few solutions, and employs preparation and reaction times that allow laxity. Polyacrylamide gels of different acrylamide concentrations with or without denaturing agents stained equally well with our protocol, indicating that the silver stain is widely adaptable to different gel types and can be used with various procedures for nucleic acid analysis. Furthermore, our simplified procedure can be used to stain RNA, proteins, and polysaccharides extending its usefulness to other biochemical analyses.

MATERIALS

Chemicals used must be of high purity analytical grade. By way of example only, the supplier name and catalog number of each chemical we use has been included. Deionized (cartridge or glass-distilled) water must be used for the preparation of all solutions. An orbital shaker is required for agitating the gels. We use the clear plastic lids from 1000-μL pipet-tip racks as trays. Our gels are typically polyester-backed 0.45-mm thick 8×10 cm gels run in Bio Rad Mini-Protean II apparatus or similar (Richmond, CA). Polyester backing film was GelBond PAG from FMC BioProducts (Rockland, ME). Reagent volumes can be scaled up for gels of larger dimensions.

1. Fixer and stop solutions: Both the fixer and stop solutions consist of 7.5% (v/v) Glacial acetic acid (Mallinckrodt #8817, St. Louis, MO). The stop solution is kept refrigerated at 4°C,

Table 1
Silver Staining Protocol

Step	Reagent	Treatment time	
		Suggested	Range
1. Fix	Fixer solution	10 min	5–30 min
2. Wash	Distilled water	2 min (3 times)	2–5 min each
3. Impregnate	Silver solution	20 min	10–60 min
4. Rinse	Distilled water	5 s	5–20 s
5. Develop	Developer solution	4 min	2–10 min
6. Stop	Stop solution	1 min	30 s–5 min

whereas the fixer can be maintained at room temperature. These solutions are stable and are usually made up in bulk (about 4 L lots).

2. Silver solution: This is made up fresh as required. Prepare about 100 mL for each 8×10 cm gel to be developed. Add 1 g/L silver nitrate (Mallinckrodt #UN1493) and 1.5 mL/L formaldehyde (Mallinckrodt Solution #5016) to water, and mix by stirring or swirling the flask. This solution is toxic, and should be handled and disposed of with care.
3. Developer solution: This is made up fresh as required allowing about 100 mL for each 8×10 cm gel to be developed. Add 30 g/L sodium carbonate (anhydrous powder #101 1873, Eastman Kodak, Rochester, NY), 3 mL/L formaldehyde (Mallinckrodt solution #5016), and 2 mg/L sodium thiosulfate (Sigma #S-7143, St. Louis, MO) to water, and mix by stirring or swirling the flask. Make sure the water is swirling when the sodium carbonate is added, or it will clump and take much longer to dissolve. The thiosulfate is most easily added as 0.5 mL/L of a 0.2 g/50 mL stock solution made fresh weekly. The developer solution should be used at about 8°C. This is conveniently done by putting the solution on ice for about 15 min prior to use.

METHOD

An optimized protocol is shown in Table 1. If polyester-backed gels are used in a flat-bottomed, straight-sided staining dish as suggested above, old solutions can be conveniently tipped off since the polyester sheet will remain in place by surface tension. Treatment times were determined using polyester-backed 0.45 mm-thick 8×10 cm gels, and may vary for gels having larger dimensions or no backing film. In general, allow more

time for larger gels and (usually) less time for nonbacked gels. For preservation, gels were washed in distilled water and dried. Gloves should be worn when handling gels to avoid staining artifacts mainly resulting from fingerprints.

NOTES

1. Nucleic acid fixation: Fixation is believed to prevent diffusion of separated nucleic acid molecules within the gel matrix, and helps remove and neutralize unwanted chemicals (like urea and buffer) that could interfere with the silver stain. We found fixation to be important for sensitivity. A minimum of 5 min of immersion in 7.5% acetic acid maintained the limit of detection of DNA fragments of various lengths, but a 10-min exposure was required for optimal image contrast.
2. Gel washing: Adequate washing after fixation (a minimum of three 2-min washes in deionized water) is necessary to remove acid and other trace substances that interfere with staining.
3. Silver impregnation: Silver concentration cannot be reduced without affecting sensitivity and contrast. A careful examination of silver impregnation times showed that optimal staining can be achieved after only 20 min. However, as little as 10 min is sufficient for high-quality staining without significant loss of sensitivity. Impregnation times greater than about 90 min can cause severe image loss.
4. Image development: Image development occurs by reduction of silver ions with formaldehyde in an alkaline environment containing sodium thiosulfate. Decreased background staining of the gel is possible and determined by the kinetics of the reduction reaction. Silver ion complexants, like sodium thiosulfate, decrease the free silver ion concentration, reduce the kinetics of reduction, and thus increase the redox potential in the surrounding matrix, minimizing background staining (*8*). Decreasing sodium carbonate concentration below the recommended levels causes higher background staining and poor image contrast. Image development occurs too quickly to control when staining is done at temperatures above 10°C, overdevelopment and browning of the gel surface are the usual outcome. We have found poor staining usually results from low-quality or old reagents. Make sure formaldehyde is stored at room temperature since cold storage will inactivate it by polymerization.
5. Stopping the reaction: When optimal image intensity is obtained, the development reaction is stopped by lowering pH.

Since development occurs quickly compared to other methods, it is best to stop the reaction as abruptly as possible to avoid accidental overdevelopment. This is best done using cold (4°C) 7.5% acetic acid. Higher acetic acid concentrations can cause image fading and should be avoided.

6. Clean up: The silver nitrate solution is toxic and should be disposed of with care. We precipitate the used silver with NaCl and accumulate it for recycling. The staining dishes need not be absolutely clean but should be wiped out after use. Wipe up any spilt silver solution immediately, or black stains will occur. Stains can usually be removed with a nitric acid rinse.
7. Gel preservation: Preservation of original gel material has many advantages. For example, DNA can be isolated from dried silver stained gels and amplified using the PCR at any time (*9*). Polyester-backed gels can be preserved for many years by drying without suffering distortion or detectable image loss. Our oldest gels (about 3 yr) are still perfectly preserved. Nonbacked gels can also be preserved, usually between plastic sheets (such as BioGelWrap; Bio-Design Inc.). However, in our experience, nonbacked gels suffer some distortion from shrinkage and handling, and are difficult to manipulate.
8. Optional destaining of overdeveloped gels: To reverse overdeveloping (Fig. 1), remove surface blemishes, or perform stain recycling (in which a second cycle of silver staining is used to enhance sensitivity), gels can be briefly destained in a photographic reducer, such as Farmer's reducer (*10*). When using Farmer's reducer (30% potassium ferricyanide, 60% sodium thiosulfate, and 10% sodium carbonate), it is important to wash the gel extensively before destaining to remove ionic silver remnants (*10*) and to exert caution in the time of exposure to the reducer (usually not more than 10 s, followed by extensive rinsing). Brief destaining can darken the bands from brown to purple shades, and thus enhance contrast.
9. Comparison with various silver staining protocols: We have compared our optimized protocol with several other established acidic and alkaline silver stains (Fig. 1) and found it offered several advantages—it had the highest sensitivity, was the fastest to perform, had relatively few steps and reagents, produced the least number of staining artifacts, and had very low background staining (Table 2). We compared our method to the diamine complex silver stain of Wray et al. (*11*), the acidic silver stain of Heukeshoven and Dernick (*10*), the acidic stain of Gottlieb and Chavco (*8*), and the cupric-silver method of

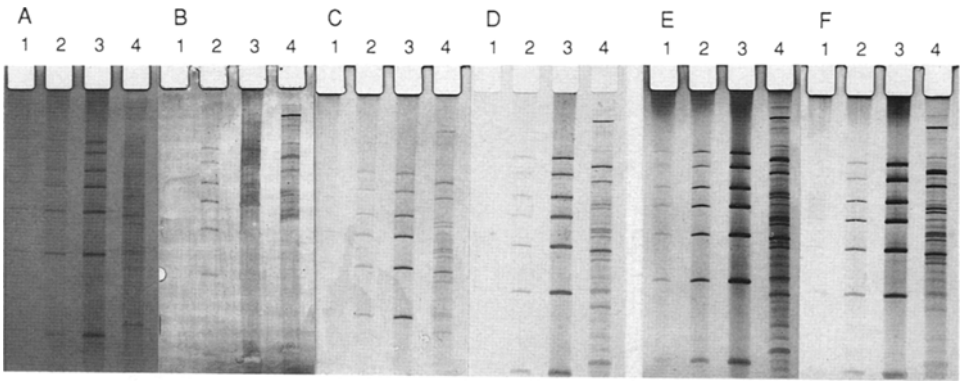

Fig. 1. Comparison of nucleic acid silver staining protocols. Biomarker low-DNA size standards having concentrations of about 10 (lane 1), 100 (lane 2), and 1000 (lane 3) pg·mm² band cross-section were used. Lane 4 shows a complex DNA profile produced from *Glycine soja* PI468-397 genomic DNA by DNA amplification fingerprinting using the oligonucleotide primer GTTACGCC. Replicate samples were run in a 5% polyacrylamide–7*M* urea gel supported by a polyester film, which was then cut into identical halves. One-half was stained with either the protocol of Budowle et al. (A), Gottlieb and Chavco (B), Switzer et al. (C), Heukeshoven and Dernick (D), or Wray et al. (not shown), and the other half stained with the simplified version of the Bassam et al. protocol (E and F) for comparison. In every instance, image development was stopped when optimal contrast between image and background was obtained, except in panel E, where the gel was overdeveloped. Panel F shows how background staining of a moderately overdeveloped gel (like in panel E) can be reduced without significant image loss by a brief (5-s) treatment of dried gels with Farmer's reducer. Gel halves were reassembled to avoid photographic bias. Each protocol was repeated at least four times, and representative results are shown. Size standards (lanes 1–3) are 1, 0.7, 0.5, 0.4, 0.3, 0.2, and 0.1 kb.

Table 2
Comparison of Nucleic Acid Silver Stain Protocols

Procedure, ref.	Sensitivity, pg·mm²	Time, min	Number of Steps	Number of Chemicals
Bassam et al. (*1*)	High (1)	26	6	5
Budowle et al. (*3*)	High (10)	37	7	6
Gottlieb and Chavco (*8*)	Intermediate (100)	70	4	10
Heukeshoven and Dernick (*10*)	Intermediate (100)	116	11	8
Wray et al. (*11*)	Intermediate (75)	145	6	8
Switzer et al. (*12*)	Intermediate (75)	110	10	13

Switzer et al. (*12*). Optimized protocols and reagents for these methods are commercially available in the silver staining kits from Polysciences, Sigma, Bio-Rad, and Boehringer-Mannheim. These stains were applied in strict observance of manufacturers directions. Similar comparison was also made to the acidic method of Budowle et al. (*3*).

The procedure of Heukeshoven and Dernick (*10*) compared favorably with ours, but had lower sensitivity and took three times as long to complete. The silver stain of Gottlieb and Chavco (*8*), although relatively fast, was the poorest in terms of background staining, sensitivity, and generation of artifacts. Moreover, staining of polyester-backed gels with this protocol produced silver mirrors at the film-gel interface. This problem was especially severe when using the alkaline silver stain of Wray et al. (*11*), which produces brown bands on an opaque mirror background. Although this method appears robust, the intense mirroring makes photography difficult, if not impossible. Finally, the acidic silver stain of Budowle et al. (*3*), used in forensic science applications, was fast and simple to perform, and had relatively good sensitivity, but poor image contrast.

Except for our procedure and those of Heukeshoven and Dernick (*10*) and Budowle et al. (*3*), scrupulous cleaning was required of staining vessels and glassware with acid or detergent. Despite these precautions, significant silver deposits accumulated on all surfaces, making handling and clean-up difficult.

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