

Staining Nucleic Acids with Silver: An Alternative to Radioisotopic and Fluorescent Labeling

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Nucleic acids separated on polyester-backed polyacrylamide gels can be detected consistently at the picogram level using a very simple acidic silver stain. The procedure is fast, has relatively few steps and reagents, and produces the least number of staining artifacts.

Introduction

Silver staining was introduced more than a decade ago as a sensitive procedure to detect trace amounts of proteins in polyacrylamide gels (1). Since then, silver staining has been perfected and extended to the study of other biological molecules that have been separated by electrophoresis in a variety of supports (2,3). Silver staining follows generally one of two methods. One uses diamine or ammoniacal silver solutions for gel impregnation and dilute acid solutions of formaldehyde for image development. The other method impregnates with silver nitrate in a weakly acidic milieu and uses formaldehyde to reduce silver under alkaline conditions. Reportedly, diamine alkaline methods are less sensitive but better suited for thicker gels, while acidic methods are fast and work best with thin gels (2).

Of the many silver staining procedures available, some provide quite sensitive and reproducible stains (4-8). During image development, almost all staining procedures reduce silver to metallic silver, which is then deposited in the immediate vicinity of the staining substratum. For optimal image contrast, the level of silver reduction in the polyacrylamide matrix must be kept to a minimum, usually by silver complexation or by "enhancing" pre-treatments of the gel matrix that precede silver impregnation. This is accomplished by appropriate modulation of the speed of the reduction process, which depends mainly on pH, the absolute and relative concentrations of silver and reducing agent, and the rate constant of the reaction. For example, complexation of silver ions decreases both silver concentration and the rate constant, allowing for selective "priming" of separated bands with metallic silver. Note that this process is not thermodynamically driven, but rather depends on the kinetics of silver reduction.

Originally, silver staining was used effectively in the detection of small amounts of nucleic acids (9-12). However, more demanding applications, such as the analysis of complex DNA profiles generated in DNA amplification fingerprinting (DAF; 13-15) and DNA sequencing (16), or the use of polyester-backed polyacrylamide gels (8,17), required a more versatile silver staining protocol. Complex nucleic acid mixtures usually are resolved in very thin polyacrylamide gels, producing bands that must be

detected reliably with high sensitivity. Backed gels have the advantage of easy handling and preservation as permanent records but are especially difficult to stain with silver, since the backing film serves as a surface for silver deposition and restricts diffusion of reagents.

Recently, we have developed a fast and highly sensitive acidic silver stain (8,18) which allows accurate detection of nucleic acids (down to 1pg DNA/mm² band cross-section) with minimum background staining. The method has fewer steps and chemicals, takes less time, and produces the least number of staining artifacts when compared to other published protocols. It has been applied successfully for the staining of DNA sequencing profiles (16) and can also be used for the staining of oligonucleotides, RNA, proteins and polysaccharides.

Here we will discuss our acidic silver stain as a versatile technique that provides unsurpassed sensitivity and reproducibility. We will also discuss the importance of the many parameters of the staining reaction. The stain currently is employed in Promega's SILVER SEQUENCE(TM) DNA Sequencing System (19) and DNA Silver Staining System (20).

A fast and simple staining protocol

The silver staining procedure is based on a photochemically-derived silver stain (11), originally designed for the staining of proteins, in which silver nitrate is the impregnating agent and formaldehyde (in an alkaline environment) is the reducer. Impregnation uses relatively low concentrations of silver in a solution containing formaldehyde. Image development occurs using formaldehyde at higher concentrations than other methods (4) and the reaction is performed at a low temperature (8-12°C) in the presence of thiosulfate. Thiosulfate chemically dissolves silver salts by complexation (4), which alters the kinetics of silver reduction and helps minimize background staining. [Figure 1](#) shows stained DNA fragments generated using DAF and separated in a polyester-backed 0.45mm thick polyacrylamide minigel.

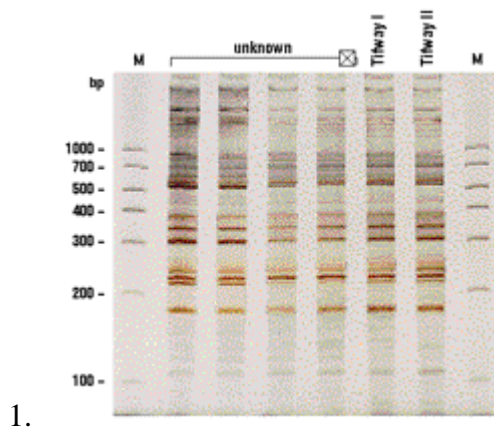


Figure 1. Silver-stained polyacrylamide gel showing DAF-generated DNA profiles of bermudagrass cultivars. The identity of DNA from unknown turfgrass samples collected from different experimental plots was inferred by direct comparison to DNA from confirmed *Cynodon dactylon* x *Cynodon transvaalensis* cultivars Tifway I and Tifway II. DNA profiles were obtained using the arbitrary oligonucleotide primer 5'-GTTACGCC-3'. The polyester-backed gel was stained using the protocol of Bassam *et al.* (8). Note the different shades of brown in the different bands, probably related to variation in DNA concentration and fragment size.

M = molecular weight markers. Courtesy of Qunyi Jiang.

After electrophoresis, glass- or polyester-backed polyacrylamide gels are fixed and stained with silver using the protocol described by Bassam *et al.* (8). Typically, gels are fixed in acetic acid for 10 minutes, washed with distilled water (3 times, 2 minutes each), impregnated with silver for 20 minutes, quickly rinsed (5-20 seconds) with distilled water, and developed at 8-12°C until optimal image contrast is obtained. Image development is stopped with the fixative, and gels are washed with water.

Table 1 shows steps and times required for the staining of backed and non-backed polyacrylamide gels of varying thicknesses using our optimized protocol. Treatment times vary for gels having larger dimensions or no backing film. In general, more time is required when staining larger gels and (usually) less time for non-backed gels.

Table 1. Protocol for Ultrasensitive Silver Staining.

Step	Reagent	Time required*
1. Fixation	Fixer solution ¹ (7.5% acetic acid)	5-30 minutes
2. Wash (3x)	Deionized water	2-5 minutes each
3. Silver impregnation	Silver solution ² (1.5g/L AgNO ₃ , 0.056% formaldehyde)	10-60 minutes
4. Rinse	Deionized water	5-20 seconds
5. Image development	Developer solution ³ (30g/L Na ₂ CO ₃ , 0.056% formaldehyde, 400µg/L sodium thiosulfate)	2-10 minutes
6. Stop	Fixer solution ¹ (7.5% acetic acid, use at 4°C)	30 seconds- 5 minutes

*Treatment times vary for gels having larger dimensions or no backing film. In general, more time is required when staining larger gels and (usually) less time for non-backed gels.

¹The fixer solution can be stored at room temperature, however, store some of the solution at 4°C for use in Step 6.

²The silver/formaldehyde solution is made up fresh as required from a silver stock solution that can be stored shielded from light for a relatively long period. Add the formaldehyde to the silver solution about 15 minutes before using.

³The developer solution is made up fresh as required, and generally used at about 8°C. Sodium carbonate solutions can be prepared in bulk and are relatively stable. Thiosulfate is added from a stock solution prepared fresh on a daily basis. Formaldehyde must be stored at room temperature since cold storage will inactivate it.

Equipment and reagent recommendations

Polyester-backed gels can be stained in straight-sided staining dishes that allow convenient discard of used solutions (the polyester sheet will remain in place by surface tension when liquid is decanted by tipping). We use the clear plastic lids from 1000µl pipette-tip racks as staining trays for polyester-backed minigels. Glass-backed gels can be stained in a similar manner. When handling sequencing gels,

glass plates can be transferred easily from one container to another. However, when handling large volumes, care must be taken to ensure appropriate temperature and moderate shaking during the critical staining steps. Solutions can be removed by aspiration when staining non-backed polyacrylamide gels.

Chemicals must be of high purity, analytical grade and solutions prepared with deionized (cartridge or glass-distilled) water. We have found that poor staining usually results from low quality or old reagents. The fixer solution can be stored at room temperature, but the stop solution should be stored at 4°C. These acetic acid solutions are stable and are usually made up in bulk. The silver/formaldehyde solution is made up fresh as required from a silver stock solution that can be stored shielded from light for a relatively long period. Silver is toxic and should be handled and disposed of with care; used silver can be precipitated with NaCl and the insoluble salt accumulated for recycling. The developer solution is made up fresh as required, and generally used at about 8°C. Sodium carbonate solutions can be prepared in bulk and are relatively stable. The thiosulfate is added from a stock solution prepared fresh on a daily basis. Formaldehyde must be stored at room temperature, since cold storage will inactivate it.

A shaker is required for agitating the gels during staining. Reciprocal shaking during development should be used to avoid fading of bands in the middle of gels. Gloves should be worn when handling gels to avoid staining artifacts from fingerprints. No special precautions are required for the glassware (beakers, flasks, staining dishes). The staining dishes need not be absolutely clean but should be wiped out after use. Wipe up any spilled silver solution immediately or black stains will occur. Stains can usually be removed with a nitric acid rinse.

Staining is sensitive and reliable

The limit of detection of double-stranded DNA upon visual inspection is approximately 1pg/mm² band cross-section (8). The limit of detection for a 100bp fragment is 0.3pg/mm², while that of a 1000bp is 3pg/mm². These detection levels are comparable to those attained with radioactive or fluorescent labels.

When compared directly with several other established acidic and alkaline silver stains (1,21-23), our protocol had the highest sensitivity, was the fastest to perform, had relatively few steps and reagents, produced very low background staining and yielded the least number of staining artifacts (18). Stains based on our procedure are available commercially as silver staining kits (e.g., Promega's SILVER SEQUENCE DNA Sequencing System, DNA Silver Staining System). The procedure was also compared to the acidic methods of Blum *et al.* (4) and Budowle *et al.* (7). While the former compared favorably with ours, it was 10 times less sensitive. The latter procedure (used in forensic science applications) had relatively good sensitivity but exhibited poor image contrast (ref. 18 and unpublished results). Unlike our procedure and other acidic silver stains (4,7,22), alkaline silver staining procedures required scrupulous cleaning of staining vessels and glassware with acid or detergent. Despite these precautions, significant silver deposits accumulated on all surfaces which made handling and clean-up difficult.

Parameters influencing the staining reaction

Several staining parameters alter the effectiveness of our silver stain. However, the protocol has been simplified by eliminating gel pretreatments prior to silver impregnation. We do not carry out any oxidation step with dichromate, hexacyanoferrate or permanganate, nor do we pretreat the gels with reducers or silver complexants. Instead, we impregnate the gel directly with a neutral silver solution. We found pretreatment steps of no obvious advantage. Similarly, special exposure to a light source was not

required.

Nucleic acid fixation

Fixation, a step believed to prevent diffusion of separated nucleic acid molecules within the gel matrix, also helps remove and neutralize unwanted chemicals (like urea and buffer). Fixation was found to be very important for sensitivity. Omitting or limiting fixation to very short exposures results in poor image development. In turn, longer exposures can result in band fading. A minimum of 5 minutes immersion in 7.5% acetic acid maintains the limit of detection of DNA fragments of various lengths.

Gel washing

This step removes acid, other trace substances and remnants of soluble gel components remaining after fixation that interfere with staining. A minimum of three 2-minute washes in deionized water appears adequate. Washes of higher or lower stringency are possible depending on backing and gel thickness.

Silver impregnation

The presence of formaldehyde in the silver solution improves sensitivity and contrast. Formaldehyde probably reduces silver at a very low rate but enough to produce initial nucleation sites around the staining substratum. These sites favor the rapid build-up of silver deposits during the development step. In this step, silver concentration cannot be reduced without affecting sensitivity and contrast. Optimal staining can be achieved after only 20 minutes. However, 8 x 10cm polyester-backed minigels require as little as 10 minutes for high quality staining without significant loss of sensitivity. Impregnation times greater than about 90 minutes can cause severe image loss.

Post-impregnation wash

While this step can be eliminated, silver remnants can cause brown precipitates in the solution during development. Alternatively, it can be replaced by a rinse in developer solution.

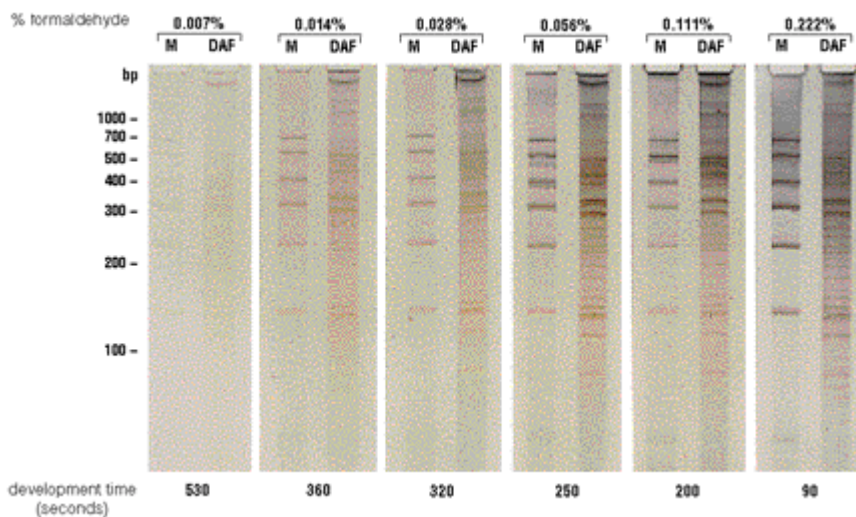
Image development

As in most silver staining procedures, image development requires an abrupt change in pH which inevitably causes the formation of insoluble silver salts. These precipitates attach to the gel surface and decrease image contrast by increasing background staining. Decreasing the concentration of silver on the surface of the gel by prior washing avoids silver precipitation but also decreases sensitivity (22). However, 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 (4). A concentration of 4 μ M sodium thiosulfate effectively reduced nonspecific background staining; higher concentrations provided no noticeable advantage (8). Decreasing sodium carbonate concentration below the recommended levels (down to about 4g/L) caused higher background staining and poor image contrast, probably by decreasing the overall rate of silver reduction.

Formaldehyde concentrations between 0.028-0.111% (by volume) provide an optimal stain ([Figure 2](#)). Lower concentrations have the general effect of reducing sensitivity. In contrast, higher formaldehyde concentrations increase sensitivity but also background staining. Higher concentrations also considerably reduce development time, making it difficult to control the staining reaction. Development time is dependent on the components of the developer solution and can vary widely from seconds to

minutes (see [Figure 2](#)). Higher formaldehyde levels produce dark brown-black bands, while low formaldehyde concentrations produce light brown colors. Obviously, the size and density of silver grains and the rate of their formation influence the color of the stained bands. In our experience, the shade of the stained nucleic acid bands is not accurately controllable. However, there is no effect on sensitivity or photographic reproduction.

The temperature of development is crucial. Typically, it must be between 8-10°C. Temperature requirements vary with gel thickness and the chemical components of the developer. However, as a general rule, image development occurs too quickly to control when staining is done at temperatures above 10°C and browning of the gel surface is the usual outcome.



1.

Figure 2. Effect of formaldehyde concentration during image development. Lanes marked "M" contain replicate samples of DNA size standards having concentrations of about 100pg/mm² band cross-section. Lanes marked "DAF" contain a complex DNA amplification fingerprinting (DAF) profile produced from *Escherichia coli* strain Smith92 genomic DNA using the mini-hairpin oligonucleotide primer 5'-GCGAAGCGAGCTG-3'. Samples were run in a 5% polyacrylamide-7M urea gel supported by a polyester film. After electrophoresis, the gel was then cut and stained using different formaldehyde concentrations in the developer solution. In every instance, image development was stopped when optimal contrast between image and background was obtained (development time is given below each treatment). Note increased browning of the gel edges with increasing formaldehyde concentration. Size standards are given in base pairs.

Stopping the reaction

When optimal image intensity is obtained, the development reaction is stopped by decreasing the pH. To avoid accidental over-development, the reaction must be stopped as quickly as possible. This is best done using cold (4°C) 7.5% acetic acid. Higher acetic acid concentrations can cause image fading.

Gel preservation

Preservation of original gel material has many advantages. Besides not requiring photography and darkroom facilities, DNA bands can be dissected out of dry silver-stained gels and amplified using PCR* at any time (14). Polyester-backed gels can be preserved for many years by air-drying without

suffering distortion or detectable image loss. Our oldest gels (about 4 years) are still perfectly preserved. Non-backed gels can also be preserved, usually between plastic sheets, but suffer some distortion from shrinking and handling and are difficult to manipulate.

*The polymerase chain reaction (PCR) process for amplifying nucleic acid is covered by U.S. Pat. Nos. 4,683,195 and 4,683,202, assigned to Hoffmann-La Roche. Patents pending in other countries.

Concluding remarks

The steps of image development in silver staining may be comparable to those of the photographic process. Crystal nuclei of metallic silver, formed initially by reduction of silver ions complexed to nucleic acids through acidic or nucleophilic groups, catalyze the selective deposition of metallic silver when adequate pH and reduction rates are achieved. We have demonstrated that silver staining of nucleic acid can be performed with unsurpassed sensitivity by tailoring only a few reaction steps and keeping the staining protocol simple. Our staining procedure will no doubt have wide applications in the detection of nucleic acids at the bench of both the molecular biologist and biotechnologist.

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Ordering Information

Product	Cat. #
SILVER SEQUENCE(TM) DNA Sequencing System	Q4130

Contains sufficient reagents for 100 sets of

sequencing reactions and staining reagents for 10 gels.

Product	Cat.#
DNA Silver Staining System	DQ7050

Contains sufficient reagents to stain 10 gels, each 31cm x 38cm x 0.4mm.

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