

The Bio-Rad Silver Stain

Introduction

Silver staining was first introduced as a general protein stain useful in polyacrylamide gel analysis in 1979 by Merrill et al.⁵⁴ This first practical PAGE silver stain was of the silver diammine type adapted from early histological silver stains. In 1981, Merrill et al.⁵⁵ introduced a faster, more reliable, and very sensitive silver stain derived from a photographic chemical process. Bio-Rad's silver stain is based on this photochemical method.

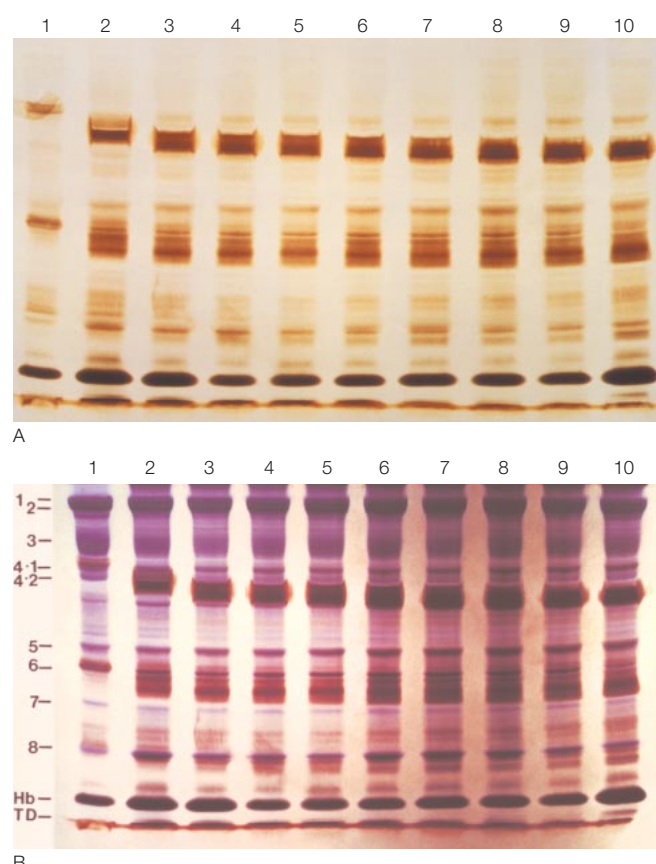


Fig. 1A and B. Differential and comprehensive visual detection of human red cell membrane proteins and sialoglycoproteins on gels stained with silver stain only (A) and with both silver/Coomassie Blue staining (B). Intact red cells were incubated with neuraminidase, and ghosts were isolated from samples taken at the indicated time intervals: 0, 5, 10, 15, 20, 25, 30, 60, 80, and 120 min (lanes 1–10, respectively). 15 μ l of packed ghost equivalents were applied to each well of an 11% (w/v) Laemmli gel.³⁹ Note the alteration in the silver stained pattern as a function of time. The silver stained bands correspond to the sialoglycoprotein bands. As can be seen, silver staining and Coomassie Blue staining are complementary, suggesting that the two stains detect different classes of macromolecules present in the gel.

The most important advantage of silver staining gels is the increased sensitivity obtained over other staining methods. Sensitivity is typically 50 times greater than obtained with classical Coomassie* Brilliant Blue R-250 staining. Increased sensitivity offers obvious advantages. For example, less sample is required when running gels. Also, analysis of dilute samples is possible. Protein purity can be assessed and contaminants detected more reliably. Silver stains detect a wider variety of macromolecules than does Coomassie Brilliant Blue R-250, including nucleic acids, glycoproteins, and lipoproteins.

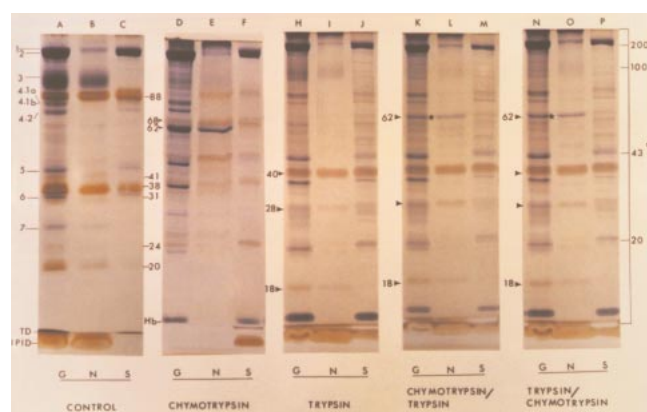


Fig. 2. Silver/Coomassie Blue double-stained gels of human erythrocyte ghosts, NaOH-stripped ghost pellets, and crude preparations of membrane sialoglycoproteins from untreated control and protease digested cells. Control cells were incubated without proteases (A–C). Test samples were erythrocytes treated with chymotrypsin (D–F), trypsin (H–J), or cells subjected to protocols of sequential digestion with chymotrypsin followed by trypsin (K–M) or trypsin treatment followed by chymotrypsin digestion (N–P).¹⁸ Ghosts (G), NaOH-stripped ghost pellets (N), and crude isolates of the membrane sialoglycoprotein (S) fractions were prepared as described. Electrophoresis was performed on a 1.5 mm thick slab gel containing isotropic 11% (w/v) acrylamide with a stacking gel of 3% (w/v) acrylamide in the Laemmli buffer system.³⁹ 15 μ l packed ghost equivalents were solubilized in Laemmli sample buffer and applied to each gel slot. After electrophoresis (12 hr at 25 mA), each gel slab was stained first with silver stain followed by Coomassie Blue staining.¹⁷ Membrane sialoglycoproteins and lipids were stained yellow. The sialoglycoproteins migrated with estimated $M_r(10^{-3}) = 88, 65, 41, 38, 31, 24$ and 20 kD (control A, C). The 62,000 M_r chymotrypsin cleavage product of band 3 was stained blue with Coomassie Blue and is marked with (*). Yellow silver-stained chymotryptic cleavage products were observed at 47 and 68 kD (D–F). Trypsin cleavage products, which stained yellow with silver, were noted at 40, 28, and 18 kD. Figure 2 reprinted in color courtesy of Biochemical and Biophysical Research Communications.

Mechanism of Silver Staining

Mechanisms and features of silver staining have been discussed in prior reviews^{51,57}. The basic mechanism occurring in silver staining of macromolecules is the reduction of ionic to metallic silver. Protein bands are imaged in the gel due to differences in oxidation/reduction potentials between sites in gels occupied by protein and adjacent sites not occupied by protein. If protein-occupied sites have the higher reducing potential, then positive images are formed. Conversely, if sites unoccupied by protein have the higher reducing potential, then negative images are formed.

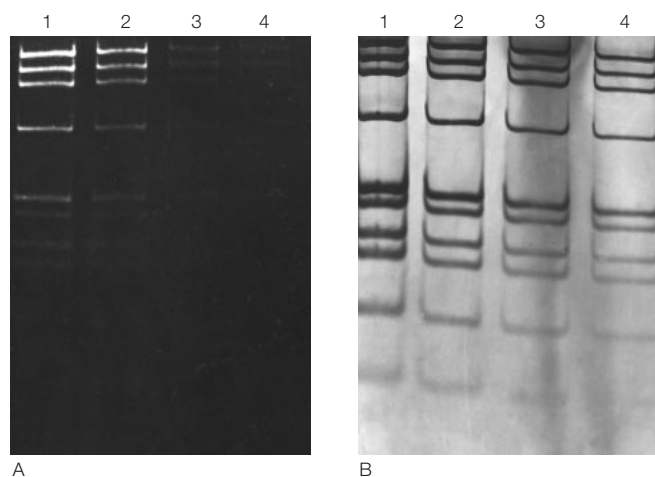


Fig. 3A and B. *HaellI* restriction digest of QX174 RF DNA electrophoresed on a 0.75 mm 6% acrylamide slab gel containing Tris-borate-EDTA (TBE), pH 8.3 buffer. Amount of digest per lane: 1, 120 ng; 2, 50 ng; 3, 25 ng; 4, 10 ng; 5, 5 ng. Gels were stained with ethidium bromide (A), or with the Bio-Rad silver stain (B).

With photographic chemical-process silver stains, the silver nitrate reacts with protein sites under acidic conditions. Subsequent reduction of silver ions to metallic silver occurs by oxidation of formaldehyde under alkaline conditions. Sodium carbonate, or another base, buffers the formic acid produced by the oxidation of formaldehyde so that the silver ion reduction can continue until the protein bands appear in the gel.

With Bio-Rad's silver stain, the formation of a positive image is enhanced by dichromate oxidation, which may convert protein hydroxyl and sulfhydryl groups to aldehydes and thiosulfates, thereby altering the redox potential of the protein. Complexes formed between the proteins and dichromate may also form nucleation centers for silver reduction. Basic and sulfur-containing amino acids appear to be strongly involved in the formation of complexes with silver ions.⁵³ This also appears to be true for Coomassie stains. The incorporation of these amino acids into peptide chains, as well as cooperative effects of several intramolecular functional groups, are probably required for reaction with silver ions.²⁹ Reduction of ionic to metallic silver is highly dependent on pH. This reduction step is accomplished by the alkaline organic development reagent.

Before a protein gel can be stained, the proteins must be fixed to minimize diffusion of molecules in the gel. Fixation also elutes substances from the gel that are likely to interfere with establishment of the oxidation/reduction potential differences and with silver reduction. Ampholytes, detergents, reducing agents, initiators or catalysts, and buffer ions (glycine, chloride, etc.) must be removed. Good-quality acrylamide and bis, free of acrylic acid and metals, are required or background will be unacceptably high. Likewise, water used in all silver stain reactions must be of 1 μ S conductance or less, and free of organic contaminants.

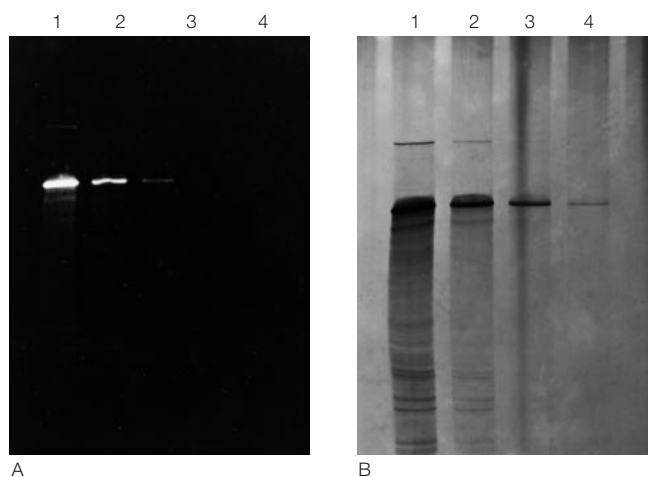


Fig. 4A and B. Sucrose gradient purified *E. coli* 16S ribosomal RNA electrophoresed on a 0.75 mm 4% acrylamide slab gel containing TBE buffer with 8 M urea. Amount of rRNA per lane: 1, 500 ng; 2, 100 ng; 3, 25 ng; 4, 5 ng. RNA sample provided by Doug Black and Dr Harry Noller, University of California, Santa Cruz. Gels were stained as described in Figure 3.

Abbreviated Procedure

An abbreviated procedure for Bio-Rad's silver stain is presented here, assuming a gel 0.75 to 1.0 mm thick. Times are longer for thicker gels and shorter for thinner gels. See the package insert for the complete procedure.

1. Fix in acid/alcohol	3 x 15 min with shaking
2. Oxidizer	5 min
3. Wash	10 min (until gel is clear)
4. Silver reagent	20 min
5. Wash	1–30 sec
6. Developer	1–10 min
<hr/>	
~90 min total	

Photochemical vs. Silver Diammine Stains

Two silver stains are generally used: diammine, or ammoniacal, types,⁵⁴ and those adapted from photographic chemical development processes.⁵⁵ Silver diammine types require that gels be soaked first in basic silver diammine, followed by acid formaldehyde image development. Chemical stains require an initial gel soak in a weakly acidic silver nitrate solution, and development in alkaline formaldehyde. Prior to the silver nitrate step, gels are primed with a reducing agent such as dithiothreitol or an oxidizing reagent such as permanganate or dichromate. Dichromate (Bio-Rad) is most desirable, because images obtained have higher sensitivity and lowest background. Dithiothreitol generates signals of lower sensitivity, while permanganate generates higher backgrounds.

Diammine silver stains suffer from many disadvantages. Reagents are not stable and must be made fresh prior to staining. Reaction products are potentially explosive. Reaction times are lengthy in comparison to photochemical methods. Histochemical silver stains cannot be combined with Coomassie stains, fluorography, or autoradiography, and gels cannot be restained in order to visualize proteins not initially detected. Staining specificity is sensitive to variations in the concentration of silver ions relative to NaOH and NH_3 , as well as to metals such as copper used in the stain, and to the acidification process used in image formation. Washes after silver diammine treatment are difficult, resulting in high background and variable sensitivity. Reproducibility and standardization of protein staining is therefore difficult.

By comparison, the photochemical method (Bio-Rad) requires only 3 reagents, which are stable on storage. It requires fewer reaction steps, and therefore is more rapid. This photochemical silver stain is sensitive, does not suffer from high background, is reproducible, and does not generate variable results when gels of different composition are stained.

Advantages of Silver Staining

Sensitive Alternative to Coomassie Blue R-250

- Dilute, unconcentrated samples can be analyzed
- Use of dilute sample avoids protein overload artifacts
- Trace samples can be analyzed
- Sample can be conserved

Useful Alternative to Autoradiography

- Faster and equally sensitive
- Less expensive
- May detect proteins not detected in radiolabeled cell lysate
- Does not require prohibitive amounts of radioactive precursors in whole animal studies
- Useful when autoradiography cannot be used

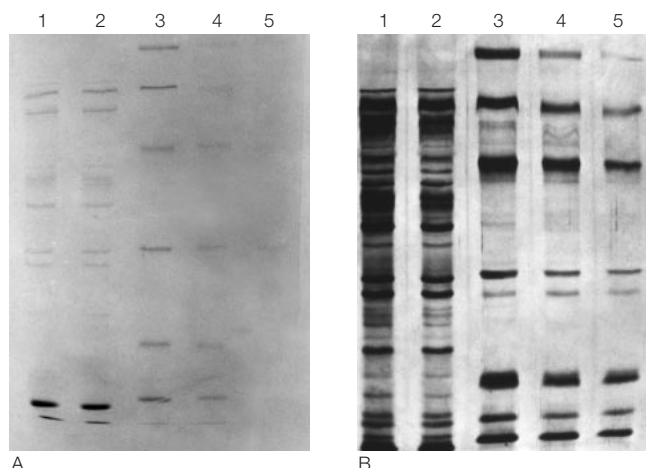


Fig. 5. SDS-PAGE according to Laemmli³⁹ stained with A, Coomassie Brilliant Blue R-250, and B, Bio-Rad's silver stain. Samples were run on 12% polyacrylamide gels 0.75 mm thick. Lane 1, bovine brain homogenate soluble fraction, 20 μl ; lane 2, bovine brain homogenate soluble fraction, 10 μl ; lane 3, Bio-Rad's SDS-PAGE low molecular weight standards, 1/40 dilution; lane 4, Bio-Rad's SDS-PAGE standards, 1/80; lane 5, Bio-Rad's SDS-PAGE standards, 1/160.

Features Unique to Photochemical Silver Stain

Simplicity

- Reliable. Most frequently quoted procedure
- Well characterized. Subject of multiple reviews^{51, 56, 57}
- Few reagents — only 3, all stable at 4°C
- Rapid. <30 min for 0.5 mm gel, <45 min for 1 mm gel, <1.5 hr for 1.5 mm gel; combined mini-gel electrophoresis and stain complete in half a day

Specificity

- Stains all types of macromolecules including glycoproteins, lipoproteins, and nucleic acids (see References)
- Stains macromolecules not stained in other silver stain methods
- Recycling gel through silver nitrate and developer detects proteins not initially visualized; for example, calmodulin^{51, 56, 57}

Universality

- Stains gels of various composition (see References) including IEF, 2-D, gradient and peptide gels, and nucleic acids in denaturing gels

Adjunct to Protein Identification and Characterization

- Protein-specific slopes (OD vs. protein concentration plot) differentiate one protein from another⁵⁷
- Colors differentiate proteins

Compatibility with Other Detection Methods

- Can be followed by autoradiography, with $\leq 2\%$ quenching^{57, 81}
- Can perform fluorography, provided gel is destained^{57, 81}
- Coomassie Blue R-250 following silver stain differentiates membrane proteins by color¹⁶⁻¹⁹
- Coomassie Blue R-250 before silver stain allows color differentiation,⁴¹ reverses negative images, and enhances sensitivity⁶¹
- Coomassie Blue G-250 before silver stain increases sensitivity 2–8 times vs. silver stain alone¹⁴

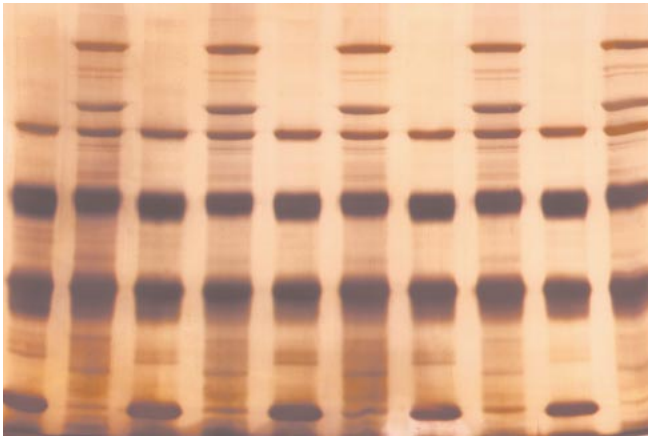


Fig. 6. Alternating high and low MW standard on a 0.75 mm gel, run at 100 V for 5 min and 200 V for 45 min in Bio-Rad's Mini-PROTEAN® II slab cell, and silver stained with Bio-Rad's silver stain kit.

Color Differentiation

- Differentiates membrane polypeptides (blue), lipid and sialoglycoprotein (yellow)¹⁶⁻¹⁹
- Differentiates polymorphisms⁴¹
- Color may be affected by charged amino side groups, bond length, and configuration⁶⁴
- Color dependent on size of silver particle: small grains red or yellow-red, large grains blue to black⁵⁷
- Color often enhanced by low concentration of reducing agent in developer, time of development, elevated temperature, or added alkali or metals in developer^{57, 64}

Sensitivity

- Typically 50x more sensitive than Coomassie Blue R-250 (0.1 ng/mm²)
- Can be as much as 200x more sensitive than Coomassie Blue R-250 (0.02 ng/mm²)⁵⁷
- Varies with protein
- Recycling or prior Coomassie staining may enhance sensitivity^{14, 57, 61}

Quantitation and Linearity⁵⁷

- Linear range 0.05–2 ng/mm²
- Quantitative comparisons limited to homologous proteins
- Optical density vs. protein concentration plot generates protein-specific plots

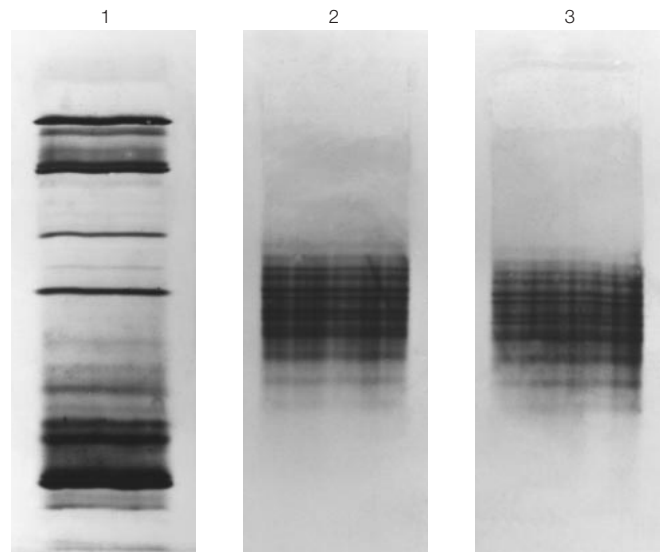


Fig. 7. Flatbed ultrathin IEF gel, pH 3–10, 5%T, 3%C stained with Bio-Rad's silver stain. Lane 1, Bio-Rad's IEF standards, is a mixture of 8 proteins, 4 of which are naturally colored; lanes 2 and 3 are goat IgG.

Nucleic Acid Stain

- More sensitive than ethidium bromide
2–5x more sensitive (<0.03 ng/mm²) for ss DNA²²
10–30x (<0.03 ng/mm²) for RNA⁴⁸
100x (0.01 ng/mm²) for ds DNA³
- Detects trace nucleic acids
- Detects small polynucleotides (10–20 bases)³; sensitivity 0.25–4 ng for DNA ≥ 271 bp
- Linear range 25–250 ng for DNA²²

References, by Categories

Methodology	References	Applications	References
Photochemical silver staining	3–5, 9, 14–17, 22, 48–51, 55–57, 65, 67, 82	Assess blotting efficiency	7, 34, 80
Coomassie G-250 stain prior to silver stain	15	Compare total protein in gels with blots	25, 32, 43, 60, 76, 80
Coomassie R-250 stain prior to silver stain	41, 61, 73	Protein purity and integrity by gel analysis	10, 35, 37, 38, 47, 59, 73
Coomassie R-250 stain after silver stain	14, 16–19	Monitor reaction products	8
Multicolor silver staining	14, 16–19, 26, 41, 61, 64	Assess purity of monoclonal antibodies	21, 77
Silver stain prior to fluorography	81	Assess enzyme purity	12, 33, 37, 47, 60, 78, 84
Silver stain prior to autoradiography	6, 81	Locate gel bands prior to band recovery	20
Gradient gels	12, 26, 34, 73, 82	Compare total proteins to those radiolabeled in vivo or in vitro	6, 58
Two-dimensional PAGE	23, 26–28, 30–32, 36, 41, 50–52, 55–57, 58, 61, 62, 68, 72, 75, 79, 81	Assess purity and molecular weight of recombinant proteins	2, 40, 42, 79
Isoelectric focusing	36, 49, 62	Precursor-product relations	16
Peptide mapping/ proteolytic digests	17, 18, 32, 45	Topological analysis of membrane surfaces	16
Quantitation	1, 15, 23, 31, 62, 75, 84	Monitor chromatography fractions	
Recycling silver stain for image intensification	51, 56, 57	HPLC	21, 34, 59, 69, 74, 85
Negative staining and image reversal	57, 61	Affinity	7, 12, 24, 46, 68, 70, 73, 76, 86
		Gel filtration/ion exchange	13, 20, 33, 42, 63, 82
		Sucrose	65, 66
		Clinical research	23, 26–28, 41, 50–52, 55–57, 61, 68, 75
		Hormones and growth, cell, and oncogenic factors	20, 24, 35, 44, 59, 63, 74, 82, 85
		Analysis of immune complexes	68
		Mutation analysis	23, 79
		Polymorphisms	23, 41
		Analysis of cerebrospinal fluid	11, 27, 28, 49, 52, 55
		Analysis of tumor cells	26, 61
		Viral protein detection	7, 13, 66, 69, 76
		Blood proteins	86
Macromolecules Stained	References		
DNA/RNA	3, 4, 22, 48, 56		
Glycoprotein	5, 40, 65, 71, 74, 86		
Sialoglycoprotein	14, 16–19, 69		
Carbohydrate	14, 46		
Glycolipid	46		
Lipoprotein	41, 75, 83		
Lipid	16–19		
Lipopolysaccharide	43		
Membrane proteins	8, 16–19, 32, 72		
Receptor proteins	1, 10, 11, 38, 46, 70, 71, 73		
Nuclear proteins	3, 58		
Total cellular proteins	18, 26, 30, 32, 36, 61, 62, 79, 81		
Polypeptides	64		
Hormones	15, 37		
Oncogenic, growth, and cell factors	20, 24, 35, 44, 59, 63, 74, 82, 85		

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