Protein Detection in Gels by Silver Staining: A Procedure Compatible with Mass-Spectrometry

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I. INTRODUCTION

Silver staining is one of the procedures, in addition to Coomassie Blue, R and G types, (Neuhoff et al., 1988) and fluorescent dyes (Steinberg et al., 1996; Patton, 2002; see also article by Patton in this volume) that are available for detecting proteins separated by gel electrophoresis. Switzer et al., introduced silver staining in 1979, a technique that today provides a very sensitive tool for protein visualization with a detection level down to the 0.3-10 ng level (Switzer et al., 1979).

The basic mechanisms underlying silver staining of proteins in gels are relatively well understood. Basically, protein detection depends on the binding of silver ions to the amino acid side chains, primary the sulfhydryl and carboxyl groups of proteins (Switzer et al., 1979; Oakley et al., 1980; Merril et al., 1981; Merril et al., 1986), followed by reduction to free metallic silver (Rabilloud, 1990; Rabilloud, 1999). The protein bands are visualized as spots where the reduction occurs and, as a result, the image of protein distribution within the gel is based on the difference in oxidation-reduction potential between the gel’s area occupied by proteins and the free adjacent sites. A number of alteration in the silver staining procedure can shift the oxidation-reduction equilibrium in a way that gel-separated proteins will be visualized either as positively or negatively stained bands (Merril et al., 1986). Silver staining protocols can be divided into two general categories: 1) silver amine or alkaline methods, and 2) silver nitrate or acidic methods (Merril, 1990). In general, the detection levels’ using the various procedures is determined by how quickly the protein bands develop in relationship to the background (e.g. signal-to-noise ratio). The silver amine or alkaline methods usually have lower background and as a result are most sensitive but require longer procedures. Acidic protocols, on the other hand, are faster but slightly less sensitive. A comparative analysis of the sensitivity of a number of silver staining procedures has been published recently (Sorensen et al., 2002; Mortz et al., 2001). Clearly, each protocol has different advantages regarding timing, sensitivity, cost, and compatibility with other analytical methods, especially mass-spectrometry (MS) a tool that is
being used in combination with gel electrophoresis or chromatographic methods for rapid protein identification (see various articles in this volume). Until recently most of the silver staining protocols used either glutaraldehyde- or formaldehyde-based sensitizes in the fixing and sensitization step, thus, introducing the chemical modifications into proteins. The utilization of those chemicals causes the cross-linking of two lysine residues within protein chain, thus, strongly affecting two most crucial steps for the subsequent MS analysis: hampering the trypsin digestion and highly reduces the protein extraction from the gel (Rabilloud, 1990).

Several modifications of the silver nitrate staining procedure have been developed for visualizing proteins that can be subsequently digested, recovered from the gel, and subjected to MS analysis (Shevchenko et al., 1996; Yan et al., 2000). In this article we describe a procedure that is slightly modified from these.

II. MATERIALS AND INSTRUMENTATION

Ultra pure water (> 18 megohm/cm resistance) for preparation of all buffers as well as during the washing steps is recommended. Use high quality laboratory reagents that can be purchased from any commonly used chemical company.

III. PROCEDURE

To achieve the best results, that is high sensitivity and low background, it is very important to follow closely the incubation time of all steps as given in the protocol.

Solutions

1. **Fixation solution**: 50% ethanol (or methanol), 12% acetic acid, 0.05% formalin. To make 1 liter, add 120 ml of glacial acetic acid to 500 ml of 96% ethanol and 500 µl of 35% formaldehyde (note: commercial formalin is 35% formaldehyde). Complete to final volume with deionized water. Store at room temperature.

2. **Washing**: 20% ethanol (or methanol). To make 1 liter, add 200 ml 96% ethanol to 800 ml of deionized water. Store at room temperature.

3. **Sensitizing solution**: 0.02% (w/v) sodium thiosulfate (Na₂S₂O₃). To make 1 liter add 200 mg of sodium thiosulfate anhydrate to small volume of deionized water, mix well and bring to the final volume of 1 liter.
4. **Staining**: 0.2% (w/v) silver nitrate (AgNO₃), 0.076% formalin. Has to be prepared fresh. To make 1 liter add 2 g of AgNO₃ to a small amount of deionized water. Add 760 µl of 35% formaldehyde. Dissolve and bring to final volume with deionized water. Pre-cool the solution at 4°C before using.

5. **Developing solution**: 6% (w/v) sodium carbonate (Na₂CO₃), 0.0004% (w/v) sodium thiosulfate (Na₂S₂O₃), 0.05% formalin. To make 1 liter add 60 g Na₂CO₃ to a small amount of deionized water and dissolve. Add 4 mg of sodium thiosulfate anhydrate to a small volume of deionized water and dissolve. Mix both solutions, add 500 µl of 35% formaldehyde and bring to the final volume with the water. Store at room temperature.

6. **Terminating solution**: 12% acetic acid. To make 1 liter, add 120 ml of glacial acetic acid to 500 ml of deionized water. Mix well and bring to the final volume with water. Store at room temperature.

7. **Drying solution**: 20% ethanol. To make 1 liter, add 200 ml of ethanol to 800 ml of deionized water. Mix well. Store at room temperature.

**Steps**

Use powder free rubber gloves throughout the procedure. Wash the gloves with water during the staining procedure. The gel fixation and washing procedure can be carried in a staining try (polypropylene trays are recommended) but make sure that these are only used for silver staining. The size of the container has to be big enough to perform the free movement of the gel during the shaking. For each step use sufficient volumes of the solutions to fully immerse the gels. Close the plastic trays by the lead or place the trays on the top of each other to protect the gels from dust. Perform all steps at room temperature, one gel per tray, placed on a shaker at a very gentle speed. Do not touch the gel with the bare hands or metal objects during the handling. Plastic or Teflon bars (or ordinary glass pipettes) can be used to handle the gel. The staining procedure can be performed on any type of the rotary shaker.

1. After electrophoresis, remove the gel from the cassette and place into a tray containing appropriate volume of fixing solution. Soak the gel in this solution app. 2 h. Fixation will restrict protein movement from the gel matrix and will remove interfering ions and detergent from the
gel. Fixation can also be done overnight. It may improve the sensitivity of the staining and decrease the background.

2. Discard the fixative solution and wash the gel in 20% ethanol for 20 min. Change the solution three times to remove the remaining detergent ions as well as fixation acid from the gel. **Note:** We recommend using ethanol solution instead of deionized water to prevent gel’s swelling. If water is used during the washing step the size of the gel can be restoring by incubation of the gel in 20% ethanol for 20 min.

3. Discard the ethanol solution and add enough volume of the sensitizing solution. Incubate for 2 min with gentle rotation. It will increase the sensitivity and the contrast of the staining.

4. Discard the sensitizing solution and wash the gel twice, 1 min each time, in deionized water. Discard the water.

5. Add the cold silver staining solution and shake for 20 min to allow the silver ions to bind to proteins. **Note:** Do not pour the staining solution directly on the gel as it may result in unequal background. Add the solution to the corner of the tray.

6. After staining is complete, pour off the staining solution and rinse the gel with a large volume of deionized water to 20-60 sec to remove the excess of unbound silver ions. Repeat the washing once more. **Note:** Washing the gel for more than one min will remove the silver ions from the gel resulting in decreased sensitivity.

7. Rinse the gel shortly with the developing solution. Discard the solution.

8. Add new portion of the developing solution and develop the protein image by incubation the gel in 300 ml of developing solution for 2 - 5 min. The reaction can be stopped as soon as the desired intensity of the bands is reached.

9. Stop the reduction reaction by adding 50 ml of terminating solution directly to the gel that is still immersed into developing solution. Gently agitate the gel during 10 min. As soon as “bubbling” of the solution is over, the development is stopped.

10. Moist gels can be kept in 12% acetic acid at 4°C in sealed plastic bags or placed in the drying solution for 2 hr prior to vacuum drying.

Figures 1A, B shows silver stained gels of total protein extracts from normal colon tissue biopsy and a breast tumor biopsy separated by 2D gel electrophoresis as described by Celis *et al.*, in this volume. Since the sensitivity of silver staining is in the same range as the modern mass
spectrometry, it makes this staining one of the most attractive technique for protein visualization before the MS analysis. Protein bands of various intensity can be excised from the gels and identify by MS analysis (see various articles in this volume).
Figure 1 2D gel of normal human colon, location 7, (A) and human breast tumor biopsy (B) separated by 2D-gel IEF electrophoresis and stained with silver nitrate. Protein spots labeled on the gel images were identified by MALDI-TOF-MS analysis. MS analysis of proteins resolved by gel electrophoresis utilizes the extraction of protein spot from the stained gel followed by trypsin digestion, measurement and database analysis.

IV. COMMENTS

When choosing the silver staining protocol it is necessary to remember that not all proteins are equally staining by this technique. Thus, several classes of highly negative charged proteins, including proteoglu cans and mucins, which contain high levels of sulfated sugar residues, and some of very acidic proteins are detected poorly by silver staining (Goldberg H.A. at al, 1997).

Note, that linear dynamic range of the stain is restricted to app. 10-fold range, thus hampering the use of this method for quantitative protein expression analysis.

Several silver staining kits that offer improved compatibility with subsequent mass spectrometric analysis are commercially available, these include: Silver Stain PlusOne; Amersham Pharmacia Biotech, Amersham, UK and SilverQuest™ Silver Staining Kit, Invitrogen, USA.

V. PITFALLS

1. To increase the sensitivity of the staining, wash the gel longer after fixation to remove all residual acid. This extra washing will reduce the background during development.

2. Development of the gel for a long period of time can decrease the yield of the peptides for subsequent mass-spectrometric analysis. This is due to the fact that mainly unstained peptides from the inner part of the gel are eluted to the solutions following “in gel” tryptic digestion” of proteins (see article in the volume).

3. Negative staining can be observed when an excess of protein is applied.

4. In some cases, artificial bands with a molecular mass of around 50-70 kDa as well as streaking or yellow background can be observed due to the presence of high concentration of reducing agents such as 2-mercaptoethanol or DTT in the sample buffer.

5. Recently it was published that the recovery of peptides from the gel, for MS analysis, can be increased by distaining of the silver stained protein bands (Gharahdaghi at al., 1999). The
unpublished observation is indicated that it will only be the case if the distaining is performed immediately after the staining procedure.

References


