Silver staining of proteins in polyacrylamide gels

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Silver staining is used to detect proteins after electrophoretic separation on polyacrylamide gels. It combines excellent sensitivity (in the low nanogram range) with the use of very simple and cheap equipment and chemicals. It is compatible with downstream processing, such as mass spectrometry analysis after protein digestion. The sequential phases of silver staining are protein fixation, then sensitization, then silver impregnation and finally image development. Several variants of silver staining are described here, which can be completed in a time range from 2 h to 1 d after the end of the electrophoretic separation. Once completed, the stain is stable for several weeks.

INTRODUCTION

Among the various methods of protein detection after electrophoresis of polyacrylamide gels, silver staining has gained wide popularity because of its sensitivity (in the very low nanogram range), because it can be achieved with simple and cheap laboratory reagents and because it does not require complicated and expensive hardware for the readout. Furthermore, silver staining, in at least some of its variants, is also compatible with downstream processing such as mass spectrometry.

The rationale of silver staining is quite simple. Proteins bind silver ions, which can be reduced, under appropriate conditions, to build up a visible image made of finely divided silver metal. However, silver staining can be a tricky business, as many artifacts and pitfalls exist. Unlike staining with organic dyes, silver staining goes against general thermodynamics. Proteins bind silver ions, and this binding decreases the reactivity of the ions. This general phenomenon explains why 'hollow' or 'doughnut' bands or spots are so commonly encountered. However, silver ion reduction is extremely self-catalytic, so that any trick that promotes silver reduction at the sites of proteins will favor sensitive and positive staining of proteins. The description and discussion of those tricks, which are part of the sensitization process, are beyond the scope of this paper and can be found elsewhere[1].

All silver-staining protocols, starting from the pioneer work[2], are made of the same basic steps, which are the following: (i) fixation to get rid of interfering compounds, (ii) sensitization and rinses to increase the sensitivity and contrast of the staining, (iii) silver impregnation with either a silver nitrate solution or a silver-ammonia complex solution, (iv) rinses and development to build up the silver metal image and (v) stop and rinse to end development before excessive background formation and to remove excess silver ion and other chemicals before further processing. Because of the variations that can be introduced at each of these steps, several dozen protocols are described in the literature, which can be somewhat confusing. It is therefore the purpose of this article to describe selected protocols that face the most commonly encountered constraints.

In the field of silver staining, two families of methods coexist, depending on the reagent used for silver impregnation. This can be either simple silver nitrate or a silver-ammonia complex. Silver nitrate stains are the simplest ones. Their compatibility with mass spectrometry is moderate but can be improved with some modifications listed below (for example, shortening the fixation time), at the expense of the quality of the stain itself. More background is obtained at high room temperatures (>30 °C). Basic proteins are less efficiently stained than acidic ones with this type of stain. This type of stain is also compatible with various electrophoretic systems (including, for, example the Tricine[3] and Bicine[4] systems) and works for most commercial gels. It is a good choice when numerous gels must be stained and when staining consistency is important.

Silver-ammonia stains are less straightforward than silver nitrate stains, but they offer more flexibility in the control of staining. They do not work properly below 19–20 °C, except when the water used for making the solutions for each step is warmed to 20–25 °C or above before use. This type of stain stains basic proteins more efficiently than acidic ones. Only the classical glycine electrophoresis system and the taurine system[5] can be used with this type of stain, which requires homemade gels[6]. Gels must be polymerized with a complex initiator system. To 1 ml gel mix, 0.7 µl TEMED, 5 µl 10% (wt/vol) sodium thiosulfate pentahydrate and 7 µl 10% (wt/vol) ammonium thiosulfate are sequentially added. The gel is then cast and polymerized for at least 1 h. Gels made in this way can be kept in a cold room for up to 1 week.

When selecting the most appropriate protocol, the experimenter must answer a few questions.

Do I want to use only precast gels, or am I ready to cast my own polyacrylamide gel?

Some very efficient silver-staining protocols (for example, those using silver-ammonia complex) require special gels that can be made easily in the laboratory but that have a limited shelf life, preventing them from being commercialized.

Will I stain 1D or 2D gels?

2D gels are often contaminated with carrier ampholytes, which are difficult to remove and give staining artifacts. Consequently, 2D gels require more rigorous fixation to thoroughly remove the ampholytes.

Do I wish to go further with my spots (for example, with mass spectrometry), or is silver staining the final step?

Fixation with aldehydes (formaldehyde, glutaraldehyde) greatly improves fixation, sensitivity and uniformity of staining. However, it precludes any further use of such stained spots.

TABLE 1 | Best silver-staining protocols for typical research situations.

Research needs	Best protocol
Fast, sensitive visualization of proteins, on only a few gels per series, no special needs afterwards	Fast silver staining (Step 1A)
Comparative studies of large series of gels over an extended time period, maximal consistency and good linearity of stain required	Long silver nitrate staining (Step 1B)
Sensitive detection required, without any quantitative analysis, but maximal sequence coverage required in subsequent analysis with mass spectrometry	Ultrafast silver nitrate staining (variation of Step 1A)
Combination of sensitivity, linearity and good compatibility with mass spectrometry	Aldehyde-free silver-ammonia staining (Step 1C)
Maximal sensitivity required, no need for any subsequent analysis	Formaldehyde-silver-ammonia staining (Step 1D)

Is downstream processing more important than silver staining itself?

Roughly speaking, there is a balance between the quality of staining itself and the yields obtained in downstream processing. Steps favoring a better image (for example, increased fixation) decrease the peptide yield in mass spectrometry.

Which parameter do I favor between speed and regularity of staining?

Generally speaking, fast protocols use short steps (1 min or less), which are difficult to make very reproducible. This may be a problem when staining consistency is a critical parameter (for example, in large series of 2D gels).

What is the quality of temperature control in the laboratory?

As silver staining is a delicate process, its is quite temperature dependent. Consequently, some protocols work poorly when it is too cold (below 20 °C), whereas other protocols work poorly when it is too hot (above 30 °C).

Depending on the answers to these questions, one protocol will be more optimal than the others. Typical situations and corresponding choices are summarized in **Table 1**. Note that not all the reagents and materials listed below are necessary for a given silver-staining protocol. Check what is needed by reading the procedure first.

General practice

Batches of gels (up to four gels per box) can be stained. For a batch of three to five medium-sized gels (for example, 160 mm × 200 mm × 1.5 mm), use 1 l of the required solution, which corresponds to a solution/gel volume ratio of 5 or more. Use 500 ml of solution for one or two gels of this size. The volumes can be adjusted according to the gel size, provided that a solution/gel volume ratio is at least 5 and that each gel floats freely in the solution. Batch processing can be used for every step longer than 5 min, except for image development, where one gel per box is required (the steps where batch processing cannot be used are stated in PROCEDURE). For steps shorter than 5 min, dip the gels individually in the corresponding solution.

The best way to change solutions is to use a rigid plastic sheet. Press the sheet on the pile of gels with the aid of a gloved hand. Incline the entire setup to allow the emptying of the box while keeping the gels in it. Pour the next solution with the plastic sheet in place, which prevents the incoming solution flow from breaking the gels. Remove the plastic sheet after the solution change and keep it in a separate box filled with water until the next solution change. Change this water after each complete round of silver staining.

When gels must be handled individually, manipulate them with gloved hands. The use of powder–free, nitrile gloves is strongly recommended, as powdered latex gloves are often the cause of pressure marks. Except for development or short steps, where occasional hand agitation of the staining vessel is convenient, constant agitation on a rocking table is required for all the steps.

Four different silver-staining protocols (A, B, C and D) are described in this paper, to improve our coverage of various experimental needs.

MATERIALS

REAGENTS

- Acetic acid (glacial)
- Ethanol ▲ **CRITICAL** 95% ethanol can be used instead of absolute ethanol without any volume correction. It is strongly recommended not to use denatured alcohol.
- Water ▲ **CRITICAL** Silver staining is very sensitive to trace impurities in water. Water with a resistivity greater than 15 MΩ cm^{-1} must be used.
- Silver nitrate ▲ **CRITICAL** This is usually used as a stock solution (typically 20% wt/vol), which must be stored in a dark place (a fridge is an ideal cold, dark place). Otherwise, a titrated silver nitrate solution (1 M, available from, for example, Riedel de Haën) can be purchased and used.
- Sodium thiosulfate: this chemical is used as the pentahydrate salt and can be purchased from various suppliers; a good pro-analysis grade should be used. ▲ **CRITICAL** It must be remembered that thiosulfate is not a very stable chemical. Upon aging of solutions or even the stock powder, a yellowish background has a tendency to increase on the gels. For convenience, a 10% stock solution can be prepared. It is stored at room temperature and kept for no longer than a week.
- Naphthalene disulfonate: this chemical exists as several isomers, either in the free acid form or in the disodium salt form; the use of 2,7-naphtalene disulfonic acid disodium salt (Acros) is recommended, but 1,5-naphtalene disulfonic acid (Aldrich) can also be used.
- Formaldehyde: a 37% or 40% stock solution is used (formalin) ! **CAUTION** Concentrated formaldehyde is toxic, and should be handled under a hood. ▲ **CRITICAL** Formaldehyde polymerizes over time and in the cold, resulting in a decrease in the concentration of active formaldehyde. The formalin solution must not be stored at low temperatures, and solution with a heavy deposit of polymer must not be used.
- Tris
- Potassium ferricyanide
- Ammonium hydrogenocarbonate
- Carbohydrazide

EQUIPMENT

- Rocking table: this should not be an orbital shaker, but rather a rocking table with a ping-pong move; its speed should be adjustable, and it should be able to operate at 30–60 strokes per minute.

- Containers: glass or plastic can be used; glass is easier to clean but heavier and more fragile, whereas plastic has the opposite characteristics; among plastics, polyethylene food boxes are recommended ▲ CRITICAL Plastic boxes should be thoroughly cleaned with acetone and then alcohol before first use to remove traces of plasticizers and unmolding agents. The bottom area of the container should be at least 20% greater than the area of the gels to be stained.
- Rigid plastic sheet: this is a very useful accessory to change the solutions without touching the gels; we use the polycarbonate sheets available from electrophoresis setups suppliers (for example, BioRad, GE Healthcare) for separating gel units in multigel casting chambers.

REAGENT SETUP

Tetrathionate sensitizing solution For a liter of solution, dissolve 50 g potassium acetate and 3 g potassium tetrathionate in 500 ml water. Add water to 700 ml, then 300 ml ethanol. ▲ CRITICAL This solution must be prepared the day of use.

Silver-ammonia solution This stock solution is prepared from silver nitrate, sodium hydroxide and ammonium hydroxide (5 M; Aldrich): here, again, the use of titrated solutions is strongly recommended. The solution is stored in the fridge and can be stable for months. Prepare the silver-ammonia solution as follows: for ~500 ml staining solution, place 475 ml water in a flask with strong magnetic stirring. First, add 7 ml 1 M sodium hydroxide, then 7.5 ml 5 M ammonium hydroxide (Aldrich) and finally 12 ml 1 M silver nitrate. A transient brown precipitate forms during addition of silver nitrate. It should disappear a few seconds after the end of silver addition. Persistence of a brown precipitate or color indicates exhaustion of the stock ammonium hydroxide solution. Attempts to correct the problem by adding more ammonium hydroxide are useless, as sensitivity drops. ! CAUTION Flasks used for preparation of silver-ammonia complexes and silver-ammonia solutions must not be left to dry out, as explosive silver azide may form. Flasks must be rinsed at once with distilled water, and used silver solutions should be put in a dedicated waste vessel containing either sodium chloride or a reducer (for example, ascorbic acid) to precipitate silver. ▲ CRITICAL The ammonia/silver ratio is a critical parameter for good sensitivity[7]. The above proportions give a ratio of 3:1, which is one of the lowest practical ratios. For special purposes—for example, when a stain with a sensitivity intermediate between Coomassie blue and classical silver staining is needed—the ammonia concentration can be increased up to 2.5-fold above the given concentrations, resulting in 'derating' of the silver staining.

Developer for silver nitrate staining (basic developer) 3% (wt/vol) potassium carbonate plus 250 µl formalin and 125 µl 10% (wt/vol) sodium thiosulfate per liter. ▲ CRITICAL Developer must be prepared the day of use, and formaldehyde should be added to the developer at most 1 h before use.

Developer for silver-ammonia staining (acidic developer) 350 µM citric acid containing 1 ml formalin per liter. Image development but also background development are much faster than with silver nitrate protocols. Thiosulfate, which is a powerful background reducer, cannot be used in the developer and must be included in the gel itself. ▲ CRITICAL Developer must be prepared the day of use, and formaldehyde should be added to the developer at most 1 h before use.

Stop solution for silver nitrate staining (Tris stop solution) 4% (wt/vol) Tris and 2% (vol/vol) acetic acid. ▲ CRITICAL Solution must be prepared the day of use.

Stop solution for silver nitrate staining (EA stop solution) 0.5% (vol/vol) ethanolamine and 2% (vol/vol) acetic acid. ! CAUTION Solution must be prepared the day of use. Do not mix the pure chemicals and dilute with water (risk of strong exothermic reaction with spillovers). First dilute ethanolamine in water, then add acetic acid. Some white fumes of ethanolamine acetate may occur at this stage, but this is without consequences.

Destaining solution for mass spectrometry Prepare a 30 mM potassium ferricyanide solution in water. Additionally, prepare a 100 mM sodium thiosulfate solution. ▲ CRITICAL Both solutions must be prepared the day of use. Just before use, mix equal volumes of the potassium ferricyanide and thiosulfate solutions. ▲ CRITICAL The resulting yellowish solution is stable and active for less than 30 min, and must be used immediately.

PROCEDURE

1| There are four possible methods to fix and stain the gel (**Fig. 1**): short silver nitrate staining (A), long silver nitrate staining (B), aldehyde-free silver-ammonia staining (C) and silver-ammonia staining with formaldehyde fixation (D). The aldehyde-free silver-ammonia staining protocol affords superior compatibility with mass spectrometry[8]. However, it is quite prone to the 'hollow spot' phenomenon, especially for acidic glycoproteins (**Fig. 2**). The silver-ammonia staining with formaldehyde fixation protocol affords the best sensitivity and uniformity of staining (acidic and basic proteins) and the least chromatism of all silver-staining protocols. Unfortunately, it is incompatible with any downstream process.

(A) Short silver nitrate staining[9,10]. ● TIMING 2–5 h

(i) After electrophoresis, fix the gels in 30% (vol/vol) ethanol, 10% (vol/vol) acetic acid for at least 30 min. ▲ CRITICAL STEP Various fixation schemes can be used, with an influence on the final result. Ultrafast fixation (one 30-min bath) improves sequence coverage in subsequent mass spectrometry, but at the expense of strong chromatism (bands or spots can be yellow, orange, gray or brown), precluding any image analysis. Longer fixation (typically three 30-min baths) gives much better staining but lower sequence coverage. However, this intermediate fixation scheme does not

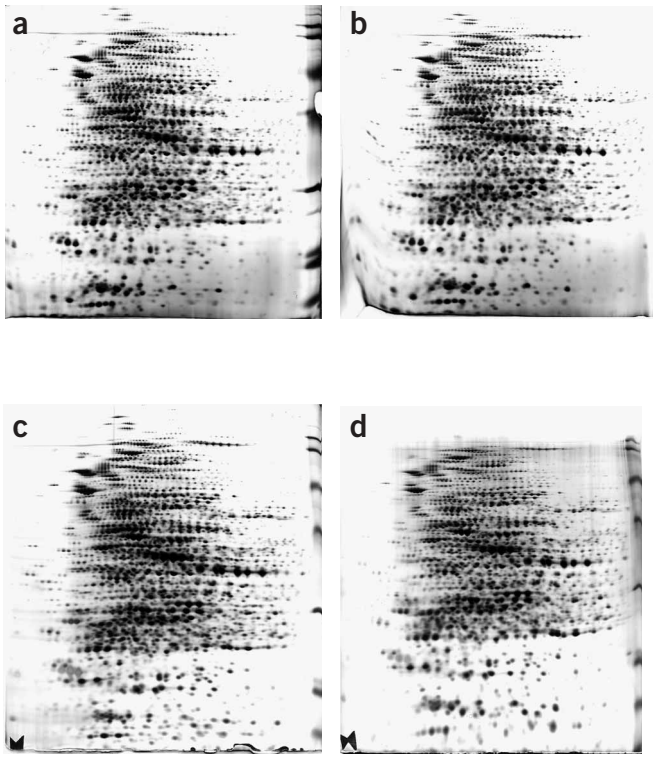

Figure 1 | Comparison of the four staining options. *Escherichia coli* total proteins (90 µg) were separated by 2D electrophoresis (pH 4–8 gradient in the first dimension, 10% (wt/vol) acrylamide in the second dimension) and detected by the four staining options described in the text. (**a**) Fast silver nitrate staining. (**b**) Long silver nitrate staining. (**c**) Aldehyde-free silver-ammonia staining. (**d**) Silver-ammonia staining with formaldehyde fixation.

adequately remove carrier ampholytes, which produce a strong background in the low–molecular weight region of the gel (**Fig. 2**). Fixation for at least 18 h is required to remove carrier ampholytes.

■ **PAUSE POINT** Fixation can be carried out for up to 24 h, with at least one solution change, which can take place when desired. However, the first bath of fixation must last at least 30 min.

(ii) Rinse the gels twice in 20% (vol/vol) ethanol, 10 min for each wash, and then twice in water, 10 min for each wash.

(iii) Sensitize the gels by soaking (one at a time only) for one minute in 0.8 mM sodium thiosulfate (0.02% (wt/vol) if the pentahydrate salt is used).

(iv) Rinse the gels twice, 1 min for each wash, in water.

▲ **CRITICAL STEP** The optimal setup for sensitization is the following: prepare four staining boxes containing, respectively, the sensitizing thiosulfate solution, water (two boxes) and the silver nitrate solution. Put the vessel containing the rinsed gels on one side of this series of boxes. Take one gel out of the vessel and dip it in the sensitizing and rinsing solutions (1 min in each solution). Then transfer to silver nitrate. Repeat this process for all the gels in the batch. A new gel can be sensitized while the former one is in the first rinse solution, provided that the 1-min time is kept (use a bench chronometer).

(v) Impregnate with 12 mM silver nitrate. Gels can turn yellow at this stage, but this does not affect the final quality.

■ **PAUSE POINT** Silver nitrate impregnation can last from 20 min to 2 h without any real change in the quality of the results.

(vi) Only one gel per tray must be used at this stage. The best setup is to arrange on the bench the box containing the gel(s) soaking in silver nitrate, one box half-filled with water, as many boxes containing developer (basic developer) as there are gels present in the same box of silver solution, and one box containing the stop solution. The stop solution, made the day of use, contains 40 g of Tris and 20 ml of acetic acid per liter. With gloved hands, rinsed with deionized water, pull one gel out of the silver solution. Dip it for 10 s in the water bath, then pull it out of the water and transfer it to the basic developer solution. A brown or gray precipitate normally develops within a few seconds when the gel is dipped in the developer. This precipitate must be redissolved by shaking of the developer gel–containing box, otherwise a particulate surface background will deposit on the gel surface. When this precipitate has redissolved, repeat the procedure for the next gel in the batch. With this protocol, the most intense bands or spots take a few minutes to appear (up to 5 min, depending on the temperature). Development can be allowed to proceed up to background development, but nothing will happen after 45 min of development. As a special trick for one application, when silver staining is used just for visualization in 1D gels before systematic band excision, the formaldehyde in the developer can be replaced by 0.5 mM carbohydrazide (final concentration in developer). The image develops and the background turns brown in a minute or two. Stop the reaction with the usual Tris-acetate solution. This protocol offers terrible staining but the best performance in the subsequent mass spectrometry[11].

(vii) When the adequate degree of staining has been achieved, transfer the gel to the Tris stop solution for at least 30 min. Up to four gels can be piled in one box of stop solution.

■ **PAUSE POINT** The time in stop solution can be extended to up to 2 h.

(viii) Wash gels in water (at least twice, 30 min for each wash). Stained gels can be stored in water for several days, but when analysis by mass spectrometry is planned, much better results are obtained if the staining, washing, spot excision and destaining are performed on the same day[11].

(B) Long silver nitrate staining[12]. ● **TIMING** Overnight plus 5 h

(i) After electrophoresis, fix the gels in 30% (vol/vol) ethanol and 10% (vol/vol) acetic acid for 1 h, then renew the fixation bath and leave them to fix overnight.

(ii) Sensitize for 45 min in tetrathionate sensitizing solution.

(iii) Rinse twice in 20% ethanol, 10 min for each wash.

(iv) Rinse four times in water, 10 min for each wash.

(v) Impregnate with 12 mM silver nitrate. Gels can turn yellow at this stage, but this does not affect the final quality.

■ **PAUSE POINT** Silver nitrate impregnation can last from 20 min to 2 h without any real change in the quality of the results.

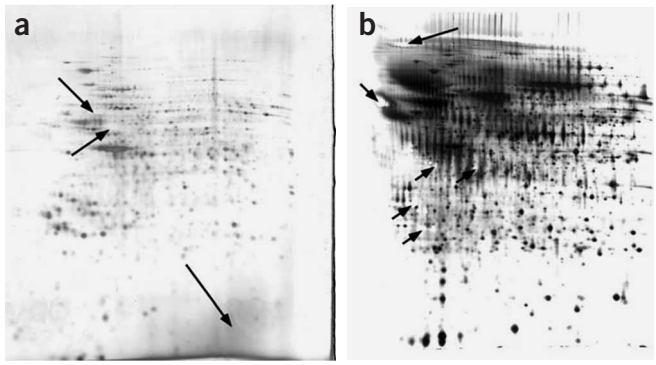

Figure 2 | Examples of staining artifacts. (**a**) HeLa total cell proteins (80 µg) were separated by 2D electrophoresis as in **Figure 1a** and detected by ultrafast silver staining using silver nitrate. Arrows indicate weakly stained spots (very yellow and thus very pale upon scanning in black and white) and the low–molecular weight gray zone corresponding to carrier ampholytes. (**b**) Monocyte-secreted proteins (150 µg) were separated by 2D electrophoresis as in **Figure 1a** and detected by mass spectrometry–compatible silver-ammonia staining. Arrows indicate some of the hollow spots (completely transparent zones on the gels) typical of this staining protocol.

(vi) Only one gel per tray must be used at this stage. The best setup is to arrange on the bench the box containing the gel(s) soaking in silver nitrate, one box half-filled with water, as many boxes containing developer (basic developer) as there are gels present in the same box of silver solution, and one box containing the stop solution. The stop solution, made the day of use, contains 40 g of Tris and 20 ml of acetic acid per liter. With gloved hands, rinsed with deionized water, pull one gel out of the silver solution. Dip it for 10 s in the water bath, then pull it out of the water and transfer it to the basic developer solution. A brown or gray precipitate normally develops within a few seconds when the gel is dipped in the developer. This precipitate must be redissolved by shaking of the developer gel–containing box, otherwise a particulate surface background will deposit on the gel surface. When this precipitate has redissolved, repeat the procedure for the next gel in the batch. With this protocol, the most intense bands or spots take a few minutes to appear (up to 5 min, depending on the temperature). Development can be allowed to proceed up to background development, but nothing will happen after 45 min of development.

(vii) When the adequate degree of staining has been achieved, transfer the gel to the Tris stop solution for at least 30 min. Up to four gels can be piled in one box of stop solution.

■ **PAUSE POINT** Time in stop solution can be extended to up to 2 h.

(viii) Wash gels in water (at least twice, 30 min for each wash). Stained gels can be stored in water for several days, but when analysis by mass spectrometry analysis is planned, much better results are obtained if the staining, washing, spot excision and destaining are performed on the same day[8].

(C) Aldehyde-free silver-ammonia staining[8,13,14]. ● **TIMING** ~5 h

(i) Fix the gels in 30% (vol/vol) ethanol, 10% (vol/vol) acetic acid and 0.05% (wt/vol) naphthalene disulfonic acid. Fixation can be carried out three times, 30 min each, but also for 30 min plus overnight, depending on the time frame and the presence of interfering compounds in the gel (see ▲ **CRITICAL STEP** below).

▲ **CRITICAL STEP** Fixation for at least 18 h is required to completely remove carrier ampholytes used in 2D gels and thus secure a clear background in the low–molecular weight region of the gel.

■ **PAUSE POINT** Fixation can be carried out for up to 24 h, with at least one solution change, which can take place when desired.

(ii) Rinse the gels in water six times, 10 min each rinse.

(iii) Impregnate for 30–60 min in silver-ammonia solution.

(iv) Rinse three times, 5 min each rinse, in water

(v) Develop image (5–10 min) in acidic developer. The most intense bands or spots should appear within 1–2 min.

(vi) Stop development in EA stop solution. Leave in this solution for 30–60 min.

(vii) Rinse with water (several changes) before drying or densitometry.

(D) High-sensitivity silver-ammonia staining with formaldehyde fixation[7,13,14]. ● **TIMING 2 h, plus overnight, plus 4 h**

(i) Immediately after electrophoresis, place the gels in water and let them rinse for 5–10 min.

(ii) Soak gels in 20% (vol/vol) ethanol containing 10% (vol/vol) formalin for 1 h.

(iii) Rinse twice, 15 min each rinse, in water.

(iv) Sensitize overnight in 0.05% (wt/vol) naphthalene disulfonate.

(v) Rinse six times, 20 min each rinse, in water.

(vi) Impregnate for 30–60 min in silver-ammonia solution.

(vii) Rinse three times, 5 min each rinse, in water.

(viii) Develop image (5–10 min) in acidic developer. The most intense bands or spots should appear within 1–2 min.

(ix) Stop development in EA stop solution. Leave in this solution for 30–60 min.

(x) Rinse with water (several changes) before drying or densitometry.

2| Destain spots before mass spectrometry. Silver staining interferes strongly with mass spectrometry analysis of spots or bands excised from stained electrophoresis gels[11]. This interference can be reduced by destaining the spots or bands before the standard digestion protocols. The destaining protocol giving the least artifacts is the ferricyanide-thiosulfate protocol of ref. 15. This protocol can be carried out on spots or bands in microtubes (0.5 or 1.5 ml) or in 96-well plates. The use of a shaking device (plate shaker or rotating wheel for tubes) is recommended. First cover the spots or bands with 0.15 ml of spot-destaining solution. The stain should be removed in 5–10 min.

3| Remove the solution and rinse the spots five times, 5 min each rinse, with water (0.15 ml per gel piece).

4| Remove the water and soak the gel pieces in 200 mM ammonium hydrogenocarbonate (in water) for 20 min (0.15 ml per gel piece).

5| Repeat Step 3.

6| Process the rinsed gel pieces, or store dry at –20 °C until use.

? TROUBLESHOOTING

The reliability and robustness of silver staining has greatly improved since the early days[2], mostly with the use of thiosulfate as a background reducer[6,8]. However, some problems occur from time to time. Typical examples are given below.

Metallic silver deposits on the surface of the gel (silver mirror)
This is most often the result of impurities coming from ill-cleaned glass plates used for gel casting. Better cleaning is required. This can also result from pressure marks (finger-like prints). In this case, use only powder-free gloves and reduce manipulation of the gels to the minimum.

In silver-ammonia staining, the solution cannot be prepared because it makes a brown-to-black precipitate
The ammonia solution is exhausted. Replace ammonia solution.

Development does not take place (no or weak image)
There is a mistake either in the developer or in the silver solution. The most common mistakes are the wrong silver dilution and use of bicarbonate instead of carbonate. If this occurs on a very precious gel, stop the reaction, rinse with several batches of water (typically ten times, 30 min each rinse) and restart from the beginning of the procedure.

Development is very slow, with weak resulting mage
In addition to the causes mentioned above, this can also result from too low a temperature. Restart as instructed above, but warm water to 18–25 °C.

In silver-ammonia staining, the gel turns brown in the silvering bath
Improper electrophoresis system. Use only the glycine or taurine systems.

A strong yellow background appears at the development stage
The thiosulfate is exhausted. Change the thiosulfate powder.

In silver nitrate staining, the developer turns black and makes a deposit on the gel
A 'black cloud' should appear when the gel is dipped in the developer. It should redissolve with shaking. If not, the thiosulfate is exhausted. Change it.

Last but not least, how to save a precious gel with a terrible background
Stop development and rinse. Then destain the gel in a solution containing 3g l^{-1} potassium ferricyanide, 6 g l^{-1} sodium thiosulfate (or, even better, ammonium thiosulfate) and 10 ml l^{-1} concentrated ammonia. This solution must be made just before use and should be green-yellow. When the background has completely disappeared, rinse with several batches of water, until the yellow color of the gel has completely disappeared. It is very likely that the bands or spots will also disappear. Rinse with water an additional five times, 30 min each rinse, then perform a fast silver nitrate staining (Step 1A), starting directly at the sensitization step (Step 1A(iii)). Expecting a decent result in mass spectrometry after such an ordeal is somewhat adventurous.

ANTICIPATED RESULTS

All the silver-staining protocols mentioned here allow protein detection in the very low nanogram range (even subnanogram)—that is, they are 30–100 times more sensitive than colloidal Coomassie blue staining[16]—with very common laboratory equipment and chemicals. The various protocols provide flexibility for many different uses, from the fast staining of minigels to large-scale staining of large series of 2D gels. However, this also means that there is no silver-staining protocol combining sensitivity, homogeneity of detection and superior compatibility with mass spectrometry.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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1. Rabilloud, T. Mechanisms of protein silver staining in polyacrylamide gels: a 10-year synthesis. *Electrophoresis* **11**, 785–794 (1990).
2. Merril, C.R. *et al.* Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211**, 1437–1438 (1981).
3. Schagger, H. & von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368–379 (1987).
4. Wiltfang, J. *et al.* A new multiphasic buffer system for sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins and peptides with molecular masses 100,000–1000, and their detection with picomolar sensitivity. *Electrophoresis* **12**, 352–366 (1991).
5. Tastet, C. *et al.* A versatile electrophoresis system for the analysis of high- and low-molecular weight proteins. *Electrophoresis* **24**, 1787–1794 (2003).

6. Hochstrasser, D.F. & Merril, C.R. 'Catalysts' for polyacrylamide gel polymerization and detection of proteins by silver staining. *Appl. Theor. Electrophor.* **1**, 35–40 (1988).
7. Eschenbruch, M. & Bürk, R.R. Experimentally improved reliability of ultrasensitive silver staining of protein in polyacrylamide gels. *Anal. Biochem.* **125**, 96–99 (1982).
8. Chevallet, M. *et al.* Improved mass spectrometry compatibility is afforded by ammoniacal silver staining. *Proteomics* **6**, 2350–2354 (2006).
9. Blum, H. *et al.* Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93–99 (1987).
10. Rabilloud, T. A comparison between low background silver diammine and silver nitrate protein stains. *Electrophoresis* **13**, 429–439 (1992).
11. Richert, S. *et al.* About the mechanism of interference of silver staining with peptide mass spectrometry. *Proteomics* **4**, 909–916 (2004).
12. Sinha, P. *et al.* A new silver staining apparatus for MALDI/TOF analysis of proteins after two-dimensional electrophoresis. *Proteomics* **1**, 835–840 (2001).
13. Mold, D.E. *et al.* Silver staining of histones in Triton-acid-urea gels. *Anal. Biochem.* **135**, 44–47 (1983).
14. Rabilloud, T. *et al.* Two-dimensional electrophoresis of human placental mitochondria and protein ientification by mass spectrometry: Toward a human mitochondrial proteome. *Electrophoresis* **19**, 1006–1014 (1998).
15. Gharahdaghi, F. *et al.* Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. *Electrophoresis* **20**, 601–605 (1999).
16. Neuhoff, V. *et al.* Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9**, 255–262 (1988).