Silver Staining
for staining proteins in polyacrylamide gels
CE1980

For research purposes only.

Kit Contents

The solutions included in the **Silver staining kit** are listed below. Sufficient reagents are supplied for 20 Mini gel according to the volume indicated at the protocols.

***Silver* kit**	**Amount**	**Symbol**
Sensitizer	40 ml	1
Stainer A	80 ml	2
Stainer B	250 ml	3
Developer	2 ml	4
Developer Enhancer	2 ml	5
Destainer A	250 ml	6
Destainer B	250 ml	7

Storage and Shipping Conditions

Silver staining kit is shipped at room temperature. Upon arriving, store the kit at room temperature. The kit is stable for 6 months when store at room temperature.

Product Qualification

The performance of **Silver staining kit** is regularly monitored. **Silver staining kit** is tested by using it for staining of GeBaGels and all standard gel and according the basic staining. Different dilutions of BSA or Lysozyme (0.1-10 ng) in 3x Sample Buffer is separated electrophoresis on a 1.4 mm thick 4-12% Gradient GeBaGel in Tris-Glycine-SDS running buffer. After staining the gel is destained using the destaining protocol.

Specifications

- Staining must detect 0.3 ng BSA
- Background must be light (yellow) and free from dark spots
- No uneven staining or contaminant bands
- Destaining of gel slice must completely destain the protein bands within 15 minutes. Destaining of gel slab is should leave 90% of the stained proteins band.

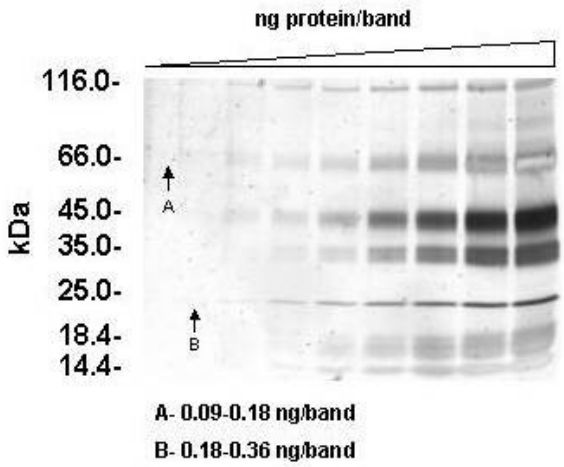

Figure 1: Example of a Silver stained gel. Using the silver staining protocol described on the Basic Staining protocol, it should be able to detect > 0.3 ng of protein and the gel should have a light background. An example of a gel stained with the Silver staining kit using the Basic Staining protocol . Twenty micro liters of unstained protein marker was loaded and run on a GeBaGel gradient 4-12% gel under denaturing conditions followed by silver staining. (A) and (B) indicate of a protein band (amount of the protein in the band are indicated below the gel).

Introduction

The Silver protein staining kit is based on the chemical reduction of silver ions to metallic silver on a protein band. This kit provides a rapid and easy method for staining proteins in polyacryamide gels and the protocol is special adapted for 1.4 mm thick GeBaGels. Silver staining allows detection of most proteins and it is 30-fold more sensitive then staining with Coomassie G-250. Also, this kit specially designed to provide sensitive staining compatible with mass spectrometry analysis.

In the table a overview of the various steps involved in silver staining of proteins:

Step	Reagent	Description
Fix	Fixative	Removes interfering ions and detergent from the gel and helps to restrict the movements of proteins out of the gel matrix.
Sensitize	Sensitizer	Increase sensitivity and contrast of the stain
Wash	Ultra pure water	Removes excess Sensitizer and rehydrates the gel for subsequent staining.
Stain	Stainer	A bind silver ions to the protein and forms a latent image.
Wash	Ultra pure water	Remove excess stainer.
Develop	Developer	Reduces silver ions to metallic silver at the protein bands resulting in development of the protein bands.
Stop	Stopper	Complexes with any free silver to prevent further reduction.

Compatibility with Mass Spectrometry Analysis

Using silver staining to stain proteins followed by mass spectrometry is a sensitive procedure for protein identification in proteomics. However, extraction of proteins from the gel is difficult, in-gel digestion of proteins with proteases (mostly trypsin) is mostly used to generate peptide fragments which analyzed using MALDI-MS to determine there exact mass. The **Silver staining kit** is specially modified to be compatible with mass spectrometry analysis by:

- Sensitizing solution DOES NOT contain glutaraldehyde. This component inhibits trypsin digestion. An important step of preparing protein for MALD-MS analysis. Moreover, this component reduces the efficiency of protein extraction from the gel by cross-linking two lysine residues.
- The kit contains also destaining solution to remove silver from the gel to improve trypsin digestion. Silver ions are inhibiting trypsin.

In the table an overview of the basic steps involved in silver staining of proteins and prepare your sample for mass spectrometry analysis:

Step	Action
1	Separate your protein sample on polyacryamide gel using any method of choice.
2	Silver stain the gel using basic or fast staining protocol using the solutions provided by the kit
3	Excise the band of interest from the gel and destain the band using destaining solution provided by the kit
4	Perform in-gel trypsin digestion and extract peptide fragments from the gel.
5	Analyze the peptide fragments by MALDI-MS

Staining Protocols

Basic Staining Protocol

Using this basic staining protocol, staining can be completed in 150 minutes (90 minutes for 1 mm thick gel).

Materials Supplied by the user:

- Ultra pure water (> 18 megohm/cm resistance recommended, see below)
- Staining tray (a polypropylene or glass tray is recommended)
- Disposable pipettes
- Clean glass bottles for reagent preparation
- Glass cylinder
- 100% acetic acid solution
- 30% ethanol (made with ultra pure water)
- 100% ethanol
- Fixative (40% ethanol, 10% acetic acid, made with ultra pure water)

Important tips for achieving optimal staining results:

- Warning: Wearing gloves is highly recommended when handling the kit contents.
- Use freshly made solutions
- Avoid cross contamination of kit solutions
- Use PTFE coated stir bars and clean glass containers to prepare reagents
- Do not touch the gel bare hands or metal objects and do not put pressure on the gels while handling or changing solutions
- Be sure that the size of the container permits free movement of the gel during shaking and complete immersion in the 100 ml solution during staining
- Be sure to wear rubber gloves that have been rinsed with ethanol
- Use clean containers and designate these containers for silver staining purposes only
- Always use ultra pure water of > 18 megohm/cm resistance for preparation of all solutions, rinsing of gels and containers. Poor quality of water may increase the background or impair protein band development
- Be sure to keep the volume of all solutions and incubation time of all steps exactly as given in the protocol. Changes in the protocol can result in high background or poor band development**.**

Preparing Before Starting

Use the reagents provided in the kit to prepare the following solutions for staining:

- **Fixing solution:** Ethanol 40 ml, acetic acid 10 ml and Ultra pure water up to 100 ml.
  It is recommended to prepare 1 L stock solution.
- **Second fixing solution:** Ethanol 30 ml and Ultra pure water up to 100 ml
  It is recommended to prepare 1 L stock solution.
- **Sensitizing solution:** Ethanol 28 ml, Sensitizer **1,** 2 ml and Ultra pure water up to 100 ml
- **Staining solution :** see preparation instruction in step 8 below.
- **Developing solution:** Developer 100 µl (**4**), Developer enhancer 100 µl (**5**) and Ultra pure water up to 200 ml

Important: You may prepare solution immediately before starting or prepare them as you proceed to the next step.

Procedure

Important: This procedure is adjusted for 8 x 8 cm GeBaGel or all standard gel running in Tris-Glycine-SDS running buffer, **1.4 mm thick**. However, with slight adjustment this protocol can be used for any protein staining in any polyacrylamide gel, just by adjusting the incubation time or the solution volume.
For any 8 x 8 cm polyacrylamide gel, **1 mm thick** follows the instruction **in parenthesis** for the incubation times.

All incubation should be performed on a rotary shaker rotating at a speed of 1 revolution/sec at room temperature.

1. After electrophoresis, remove the gel from the cassette and place it in a clean staining tray of the appropriate size. Rinse the gel briefly with ultra pure water several times.
2. Fix the gel in 100 ml of fixing solution for 25 minutes (or 20 minutes for 1 mm thick gel) with gentle rotation. If you are using Tricine gel, incubate the gel in fixative for 1 hr.

   Important: The gel can be stored in the fixative overnight if there is not enough time to complete the staining protocol. Longer fixing times may improve the sensitivity and background staining in some cases.
3. Decant the fixing solution and wash the gel in Second fixing solution for 15 (or 10) minutes.
4. Decant the Second fixing solution and add 100 ml of Sensitizing solution **(1)**. Incubate the gel in the Sensitizing solution **(1)** for 30 (or 10) minutes.
5. Decant the Sensitizing solution **(1)** and wash the gel in 100 ml of Second fixing for 15 (or 10) minutes.
6. Wash the gel with 100 ml ultra pure water for 15 (or 10) minutes.
7. Repeat step 6 once more time.
8. Decant the ultra pure water and prepare 100 ml Staining solution. Take 12.5 ml of Stainer B **(3)** and put in a chemical-cup. Add 4 ml of Stainer A **(2)** drop wise while stirring to Stainer B**(3)**. Add ultra pure water up to 100 ml. Incubate the gel in the Staining solution for 30 (or 15) minutes.

   Important: Prepare the Staining solution 5 minutes before using. The Staining solution must be fresh.
9. Decant the Staining solution and wash the gel with 100 ml of ultra pure water for 1 minute.

   Important: Washing the gel for more than 1 minute can remove silver ions from the gel and result in decreased sensitivity.
10. Decant the ultra pure water. Incubate the gel in 200 ml of Developing solution for 2-15 minutes until bands start to appear and the desired band intensity is reached.

    Important: Over develop will cause to strong dark yellow background.
11. Once the appropriate staining intensity is achieved, immediately add 10 ml of 100% acetic acid directly to the gel still immersed in Developing solution. Gently, agitate the gel for 15 minutes.
12. Decant the stopped solution and wash the gel with 100 ml of ultra pure water for 10 minutes.

If you need to destain the entire gel slab for reducing background, see **Destaining Protocol for gel slab.**

If you need to destain the gel slices containing proteins for mass spectrometry analysis, see **Destaining Protocol for gel slice.**

Silver staining after Coomassie Blue staining (CooBlue #UP47255A)

After destaining the Coomassie Blue staining, thoroughly rinse the gel in ultra pure water for 15 minutes with gentle agitation. Proceed with silver staining at the fixing step (step 2) using the basic or fast staining protocols.

Drying the Gel

You may dry the silver stained gel by vacuum drying or by air-drying. We recommend using Crack Free Solution for drying gel without cracks. Prior to drying the gel, wash the gel in ultra pure water for 15 minutes with gentle agitation to remove the Stopper solution. If using vacuum drying, please follow the manufacturer's instruction.

Fast Staining Protocol

The fast staining protocol is a modification of the basic staining protocol. This method uses microwave oven to rapidly silver stain protein gels. This staining protocol can be completed in 120 minutes.

Very Important note: Please use caution while performing the fast staining protocol using a microwave oven. Do not overheat the staining solution. Some of the staining solutions contain alcohol and alcohol fumes are highly flammable. Do not heat the staining solutions in any microwave oven that is not well ventilated or which can generate sparks. Placing a lid loosely over the staining container may minimize fumes.

Materials Supplied by the user:

- Microwave able staining tray
- Microwave oven (700-1200W)
- Ultra pure water (> 18 megohm/cm resistance recommended, see below)
- Staining tray (a polypropylene or glass tray is recommended)
- Disposable pipettes
- Clean glass bottles for reagent preparation
- Glass cylinder
- 100% acetic acid
- 30% ethanol (made with ultra pure water)
- 100% ethanol
- Fixative (40% ethanol, 10% acetic acid, made with ultra pure water)

Preparing Before Starting

See protocol of basic stain protocol

Procedure

1. After electrophoresis, remove the gel from the cassette and place it in a clean microwave able staining tray of the appropriate size. Rinse the gel briefly with ultra pure water for several times.
2. Place the gel in 100 ml fixing solution and microwave at high power (700 watts) for 30 seconds. Remove the gel from the microwave and gently agitate for 10 (or 5) minutes at room temperature. Decant the fixing solution.
3. Wash the gel with 100 ml of second fixing solution in a microwave at high power for 30 seconds. Remove the gel from the microwave and gently agitate it for 10 (or 5) minutes at room temperature on rotary shaker. Decant the second fixative solution.
4. Add 100 ml of Sensitizing solution **(1)**. Microwave at high power for 30 seconds. Remove the gel from the microwave and place it on a rotary shaker for 10 (or 2) minutes at room temperature. Decant the Sensitizing solution.
5. Wash the gel in 100 ml second fixative solution. Microwave at high power for 30 seconds. Remove the gel from the microwave and gently agitate it for 5 (or 2) minutes at room temperature. Decant the second fixative solution.
6. Wash the gel twice in 100 ml ultra pure water. Microwave at high power for 30 seconds. At each wash step, remove the gel from the microwave and gently agitate it for 5 (or 2) minutes at room temperature.
7. Decant the ultra pure water and prepare 100 ml Staining solution. Take 12.5 ml of Stainer B **(5)** and put in a chemical-cup. Add 4 ml of Stainer A **(4)** drop wise while stirring to Stainer B. Add ultra pure water up to 100 ml. Incubate the gel in the Staining solution for 30 minutes at room temperature. **Do Not Microwave.**

   Important: Prepare the Staining solution 5 minutes before using. The Staining solution must be fresh.
8. Wash the gel with 100 ml of ultra pure water for 20-60 seconds. Do not wash the gel for more than a minute.
9. Decant the ultra pure water. Incubate the gel in 200 ml of Developing solution **(4)** for 2-15 minutes at room temperature until bands start to appear and the desired band intensity is reached. **Do Not Microwave.**
10. Once the appropriate staining intensity is achieved, immediately add 10 ml of 100% acetic acid directly to the gel still immersed in Developing solution. Gently, agitate the gel for 15 minutes.
11. Decant the Stopper solution and wash the gel with 100 ml of ultra pure water for 15 minutes.

If you need to destain the entire gel slab for reducing background, see **Destaining Protocol for gel slab.**

If you need to destain the gel slices containing proteins for mass spectrometry analysis, see **Destaining Protocol for gel slice.**

Destaining Protocol

A high background of silver on over stained gels may be partially corrected by using the method below. Also, when preparing samples for mass spectrometry analysis it is important to remove silver ions from protein bands before performing in-gel trypsine digestion.
A destaining protocol using the two Destainer solutions provided in the kit to effectively remove silver ions from the gel provided below.

Destaining of gel slab

Important: Destain with a freshly prepared solution consisting of equal volumes of Destain A (6) and Destain B (7) immediately prior to use diluted by ultra pure water. Destainer solutions A and B cannot be stored for long periods once they are mixed. Do not completely destaining the gel.

12. After silver staining of the gel, wash the gel thoroughly with ultra pure water for 10 minutes.

13. Add 12.5 ml of Destain A **(6)** and 12.5 ml Destain B **(7)** and then ultra pure water up to 100 ml to the gel slab and gently, agitate the gel till the wanted results.

Important: Do not over destain. The destaining process is continue to occur also during washing with ultra pure water.
When mixing the Destain A and to Destain B the solution should change the color from blue to no color.

14. Decant the Destain solutions and wash with ultra pure water for 40 minutes. Change water several times during this step.

Important: Document the destained gel by photograph before continua.

15. Decant the ultra pure water and stop the reaction by adding 100 ml of 10% acetic acid in water for 15 minutes with gentle agitation.

16. Decant the 10% acetic acid in water and wash the gel extensively for one hour with number of water changes.

Destaining of protein in gel slice (preparing sample for mass spectrometry)

17. After silver staining of the gel, wash the gel thoroughly with ultra pure water.

18. Carefully excise the band of interest using a clean and sharp scalpel and place it into a 1.5 ml sterile microcentrifuge tube. Excise another piece of gel of the same size from a blank region of the gel and place it into another sterile microcentrifuge tube. This will be used later as a control for trypsin digestion.

19. Add 50 µl of Destain A **(6)** and 50 µl Destain B **(7)** to each microcentrifuge tube.

20. Mix the contents of the tubes thoroughly and incubate for 15 minutes at room temperature. The gel pieces will slowly settle to the bottom.

21. Carefully remove the supernatant using a clean pipette tip.

22. Add 200 µl of ultra pure water to the tube and mix thoroughly. Incubate for 10 minutes at room temperature.

23. Repeat steps 5-6 at least two times. Proceed to trypsin digestion.

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Preparing Sample for Mass Spectrometry Analysis

Once you have completed silver staining and destaining of your gel, you are ready to prepare your protein sample from the gel for mass spectrometry analysis. In this protocol we provides some guidelines for trypsin digestion and sample preparation for MALDI-MS.

Materials Supplied by the user:

- Sequencing grade trypsin
- 50 mM ammonium bicarbonate
- 1.5 ml sterile microcentrifuge tunes
- Water bath set at 37ºC
- SpeedVac
- 50% acetonitrile containing 0.1% trifluoroacetic acid
- 30% ethanol (made with ultra pure water)
- 100% ethanol
- 100 mM ammonium bicarbonate containing 30% acetonitrile

Trypsin Digestion

A general protocol for in-gel trypsin digestion is provided below. However, you may use any other method of choice or a method recommended by your protein core facility.

24. Dehydrate the gel band and the control gel band in 100% methanol for 5 minutes at room temperature.
25. Rehydrate the gel band in 30% methanol for 5 minutes.
26. Wash the gel band twice in ultra pure water for 10 minutes.
27. Wash the gel band three times with 100 mM ammonium bicarbonate containing 30% acetonitrile for 10 minutes. After the last wash, cut the gel into small pieces. Wash the gel pieces in ultra pure water.
28. Dry the gel pieces in SpeedVac for 30 minutes.
29. Resuspend the gel pieces in 59 mM ammonium bicarbonate. Add approximately 5 µl buffer per mm^2 gel. Be sure to have enough buffer to cover the gel pieces.
30. Add 5-10 ng/µl trypsin and incubate overnight at 37ºC.
31. Centrifuge at maximum speed in a microcentrifuge for 1 minute and transfer supernatant to sterile microcentrifuge tube using a clean pipet tip.
32. Extract peptides from the gel with 10-20 µl 50% acetonitrile containing 0.1% trifluoroacetic acid at room temperature. Combine this extract with the supernatant from step 8.
33. Concentrate the sample from step 9 to 4-5 ml using SpeedVac and proceed to MALDI-MS analysis. Be sure to include the control sample for MALDI-MS analysis.

MALDI-MS Analysis

The choice of matrix and the amount of sample needed for mass spectrometry analysis depends on the technique used for analysis and the individual protein sample. Basic guidelines for sample preparation are given below. For more details on sample preparation, please contact your mass spectrometry facility or core protein facility.

- Most commonly used matrices include: alpha-cyano-4-hydroxy cinnamic acid, sinapinic acid or 2, 5 dihydroxybenzoic acid.
- The sample should preferably be in ultra pure water, methanol or acetonitrile
- The sample must be in <10mM buffer or salts
- Sample concentration of 20-50 µM in a total volume of 10 µl.

Troubleshooting Guide

Problem	Cause	Comments and Suggestion
Dark or uneven background	Poor water quality	Use ultra pure water of >18 megohm/cm resistance
	Staining trays not clean or containing solutions left over from prior silver staining	Use staining trays dedicated for silver staining. After silver staining, wash trays with soap and water, and rinse them with ultra pure water.
	Improper washing done between steps	Do not skip or reduce any washing steps. If necessary increase immersing time. Alternatively, grater amount.
	Gels are bent or torn	Remove the gels carefully from the cassette after electrophoresis making sure that the gels do not tear. Be careful during handling of the gel.
	Gels are not completely submerged during staining	Be sure to completely immerse gels in staining solution and perform all steps using a rotary shaker for even staining
Poor band development or low sensitivity	Loss of silver ions from the gel	Limit the wash after staining to exactly 1 minute.
	Stainer or developer solution not prepared properly	Make sure that the solutions are prepared correctly using ultra pure water.
	Low protein load	Increase the amount of protein load. Be sure to have at least of 1 ng protein on the gel.
	Short immersing time in the step of sensitizing	Increase immersing time in sensitizing solution.
Fogging appearing during stopping the destaining	Destaining solution was not remover completely	Remove destaining solution completely by using ultra pure water washing before stopping the destaining with 10% acetic acide.
Stained gels are too dark	Stopper not added to the gel at the appropriate time	Be sure to add the stopper slightly before desired stain intensity is reached.
	Protein is overload	Decrease protein load on the gel
Large dark spots or fingerprints on the gel	Improper gel handling	Always wear gloves while handling gels. Do not apply pressure on the gels while handling.
Presence of a 50-68 kDa band across the gel	Keratin contamination	Wear gloves all times during electrophoresis and staining steps. Rinse all wells of the gel with ultra pure water before sample loading.
Longer time for band development resulting in dark background	Low protein load	Increase the amount of protein load. Be sure to have at least 1 ng protein on the gel.
	Some proteins may need longer fixing time	Increase the time for fixing the gel to 2 hours or overnight.
Negative staining	Protein band is overloaded	Decrease load per band

Related products

Product	Contents	Cat. No.
other electrophoresis gels stains		
CooBlue Instant Stain	500 ml solution	47255A
CooBlueFX Instant Protein Gel	500 ml solution	G4562A
Protein Membrane Reversible Staining	100 ml solution	20078A
Silver Stain Kit	Makes 2.5L reagents to stain 25-50 mini-gels (10x10x0.75cm)	T08860
ProSave Protein gel stain	5min procedure, fully reversible in 5min, very sensitive	BP7121
electrophoresis gels		
GeBaGel 10 %	8 GeBaGels, 10%, GeBA Sample Buffer	BI9690
GeBaGel 12 %	8 GeBaGels, 12%, GeBA Sample Buffer	BI9700
GeBaGel 4-12 %	8 GeBaGels, 4-12%, GeBA Sample Buffer	BI9710
GeBaGel 8-16 %	8 GeBaGels, 8-16%, GeBA Sample Buffer	BI9720
GeBAflex-tube **kits for extraction and dialysis of proteins from electrophoresis gels**		
Mini GeBAflex-tube (30)	30 GeBAflex-tube (volume 10-250 µl) of 6000-8000 cut-off, supporting tray, floating rack	AZ3893
Midi GeBAflex-tube (30)	30 GeBAflex-tube (volume 50-800 µl) of 3500 cut-off, supporting tray, floating rack	U27073
Maxi GeBAflex-tube (15)	15 GeBAflex-tube (volume 0.1-3 ml) of 12000-14000 cut-off, supporting tray, floating rack	AA7411
Mega GeBAflex-tube (10)	10 GeBAflex-tube (volume 3-20 ml) of 12000-14000 cut-off, supporting tray, floating rack	AZ3903
4 different sizes as indicated above	GeBAflex-tubes are available in 1,000, 3,500, 6,000-8,000, 12000-14000, 25000 and 50,000 MWCO.	
blotting reagents		
Antibody Stripping Buffer	500 ml solution	L7710A
Immunoprecipitation IpeX kit	10 IPeX spin columns, 10 collection tubes, beads, buffers	BI4211

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