Enhanced Colloidal Gold Total Protein Detection Kit

Instruction Manual

Catalog Number 170-6517

For Technical Service
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Section 1
Introduction

Silver staining is recognized as the most sensitive method for detecting proteins in polyacrylamide electrophoresis gels.[1] Researchers performing blotting applications have long wanted to obtain similar detection sensitivities for protein samples bound to nitrocellulose membranes. Bio-Rad's Enhanced Colloidal Gold Total Protein Detection Kit will identify protein bands containing as little as 400 picograms of blotted material following electrophoretic transfer, while the detection sensitivity is at the nanogram level for the same proteins in a silver stained polyacrylamide gel.

After electrophoretic transfer to nitrocellulose membrane, total protein staining is required to identify the protein bands visualized by an immunoassay. Classical methods for detecting proteins on nitrocellulose membrane employ anionic dyes such as amido black or Coomassie blue. There are two problems associated with using anionic dyes: they provide poor sensitivity, and they require use of methanolic solutions which cause nitrocellulose membrane to shrink. Once the membrane shrinks, it is difficult to match the pattern of the total protein stain with the results of an immune detection assay. Bio-Rad's colloidal gold total protein detection method solves both of these problems. The method provides high sensitivity and produces total protein stained blots that are identical in size to the immunostained membrane.

The Enhanced Colloidal Gold Total Protein Detection Kit provides the key reagents for sensitive identification of proteins on nitrocellulose membranes. The kit includes the Colloidal Gold Total Protein Stain, Tris, Tween-20, a Gold Enhancement Kit, and a detailed instruction manual. The Colloidal Gold Total Protein Stain is a gold sol which has been optimized for rapid, sensitive assays.[2] Proteins on the membrane surface stain dark red following incubation of the membrane with the colloidal gold solution. The Gold Enhancement Kit increases the assay sensitivity by depositing shells of metallic silver on top of the gold particles present on the membrane surface.[3] The silver metal turns the protein bands black, producing sharper contrast and increased detection sensitivity.

The basic assay procedure is simple and straightforward. It includes washing the membrane with a Tris/Tween-20 solution, incubating the membrane with the Colloidal Gold Total Protein Stain, and enhancing the gold stain with the Gold Enhancement Kit. The complete assay can be performed in 2–3 hours. The result is a sensitive total protein stain of the blotted membrane that can be directly compared to results obtained with Immun-Blot® assay kits.

1.1 Materials Provided in the Enhanced Colloidal Gold Total Protein Detection Kit

Catalog Number	Product Description	Quantity
170-6527	**Colloidal Gold Total Protein Stain**	500 ml
170-6538	**Gold Enhancement Kit,** containing silver lactate, monohydrate, 5 g; hydroquinone, 50 g; citric acid, anhydrous, 250 g; citric acid, trisodium salt, 250 g; fixing solution, 16 oz	1 kit
170-6531	**Tween-20**	100 ml
161-0715	**Tris**	100 gm

1.2 Reagent Storage and Stability

The Enhanced Colloidal Gold Total Protein Detection Kit components should be stored as follows:

Reagent	Temperature	Shelf Life
Colloidal Gold Total Protein Stain*	4 °C	6 months
Tween-20	23–25 °C	>1 year
Tris	23–25 °C	>1 year
Gold Enhancement Kit** (all components)	23–25 °C	>1 year

***Note:** Do not freeze the Colloidal Gold Total Protein Stain.

****Note:** The Gold Enhancement Kit components silver lactate and hydroquinone should be protected from light and kept tightly sealed.

1.3 Required Reagents Not Included With the Kit

1. Sodium chloride (NaCl)-ACS Analytical Reagent Grade.

Section 2
Enhanced Colloidal Gold Total Protein Detection Assay

Before beginning, read through the entire procedure.

2.1 Colloidal Gold Total Protein Stain

The following procedure is sufficient to assay one 15 x 15 cm nitrocellulose membrane in a 20 x 20 cm incubation vessel using 50 ml of the Colloidal Gold Total Protein Stain. This volume of gold sol should be adjusted to match the size of the incubation vessel and specific membrane being stained. (It is advisable to use the Colloidal Gold Total Protein Stain at a volume of approximately 0.2 ml per cm^2 of membrane.) Perform all wash and incubation steps at room temperature. Continuously agitate the membrane on a rotating shaker platform during all wash and incubation steps. Make sure that the membrane is completely immersed in solution during the entire assay.

1. Prepare:

   Tween-20, Tris buffered saline, 1x TTBS, 2L:

   (20 mM Tris, 500 mM NaCl, 0.3% Tween-20, pH 7.5)

   Add 4.84 g Tris base and 58.44 g NaCl to 1.9 liters of distilled, deionized water. Adjust the pH to 7.5 with HCl. Add 6 ml of Tween-20 and adjust the final volume to 2 liters.

   Note: TTBS can be made in advance and stored as a stock solution.

2. Bind proteins to a nitrocellulose membrane by one of the blotting methods described below. Always wear gloves or use forceps when handling membranes.

   a. **Electrophoretic blotting.** Electrophoretic transfer of proteins from a gel support (*i.e.*, SDS-PAGE gel, IEF gel, or native gel) to nitrocellulose membrane is best performed using the Trans-Blot® cell, Trans-Blot SD Semi-Dry transfer cell, or Mini Trans-Blot® cell. Follow the protocol outlined in the instrument instruction manual for proper electrophoretic transfer conditions.

   b. **Dot-blotting.** Cut the nitrocellulose membrane to the appropriate size (*e.g.* 0.8 x 9.2 cm strips or 3 x 5 cm rectangles). It is advisable to draw a 1 x 1 cm square grid on the membrane with a soft pencil. Next, wet the membrane by slowly sliding it at a 45° angle into Tris buffered saline (step 1) without Tween-20 (TBS) and allow it to soak for 10–15 minutes. Remove the membrane from TBS and dry it on filter paper for 10–15 minutes. Apply 1–2 µl of the protein sample to each grid square using a syringe or a variable pipette. Displace the sample to the tip of the syringe or pipette as a drop and gently touch it to the surface of the membrane. After applying the sample, air dry the membrane on filter paper for approximately 15 minutes. If the protein sample is very dilute, it is possible to apply successive aliquots at the same spot; always let the previous aliquot dry completely before applying the next one. In all cases,

the membrane should be allowed to dry completely (approximately 15 minutes) before continuing to Step 3.

c. **Microfiltration blotting.** The Bio-Dot® or Bio-Dot SF apparatus is a vacuum manifold instrument that simplifies the dot-blotting process for rapid, reproducible spotting different samples to one nitrocellulose membrane. All sample application and wash steps are conveniently carried out in the apparatus.

3. Following the binding of proteins to the nitrocellulose membrane, transfer the membrane to an incubation vessel and wash for 20 minutes with 100 ml of the TTBS solution. Discard the solution and repeat the wash two more times.
4. Discard the last TTBS wash solution and add 100 ml of distilled, deionized water to the incubation vessel. Rinse the membrane for 2 minutes. Discard the water and repeat the water rinse step two more times. The water rinse is critical to remove all salts that might interfere with the colloidal gold staining.
5. Add enough Colloidal Gold Total Protein Stain to the vessel to completely cover the membrane. Incubation times will vary with the concentration of protein present on the membrane. Concentrated protein bands will begin to appear in minutes, and all bands should be visible in 1–2 hours. Overnight incubations might increase assay sensitivity, but there is also a possibility that background staining will increase.
6. When staining is satisfactory, discard the gold sol. Rinse the membrane for 1 minute in 100 ml of distilled, deionized water. Decant the water and repeat the rinse step two more times.
7. At this point, the colloidal gold staining procedure is completed. The red color of the stained bands is stable and will not fade. If better sensitivity is desired, continue the assay by following the instructions for the gold enhancement procedure in Section 2.2. If the results are adequate, and enhancement is not necessary, air dry the membrane on filter paper. Because dried membrane is very fragile, it is best stored between two sheets of polyester film.

   Note: Small amounts of protein dotted on the border of the membrane following electrophoretic transfer, but prior to the TTBS washes, will develop color during the staining procedure. This is an excellent check on the operation of the assay and will help in gauging the rate of color development.

2.2 Gold Enhancement

It is advisable to use all solutions in volumes of at least 0.5 ml/cm^2 of membrane. Larger volumes may be used for convenience. It is best to perform the silver enhancement in a glass dish or disposable container (such as a weigh boat) to insure easy clean-up of any precipitated silver. Several membranes can be treated in the same vessel. Perform all wash and incubation steps at room temperature with continuous agitation on a rotating shaker platform. Make sure that the membrane is completely immersed in solution during the entire assay. The silver enhancement step should be performed in the dark to reduce background staining.

1. Prepare the following solutions:

   Citrate buffer*, 1x, 1 L:
   (0.2 M citrate buffer, pH 3.7)

   Dissolve 27.0 g of citric acid and 22.0 g of sodium citrate in 1 liter of distilled, deionized water. The pH should be approximately 3.7. If the pH is above 4.0, the solution should be discarded.

   Enhancement solution, 1x, 100 ml:

   a. Dissolve 0.85 g hydroquinone in 90 ml of citrate buffer (above).

   b. Immediately prior to enhancement, dissolve 0.11 g of silver lactate in 10 ml of distilled, deionized water by vortexing the solution. Mix the dissolved silver lactate solution with the 90 ml of hydroquinone solution. (Silver lactate is highly sensitive to light. Protect this solution from extended exposure to light.)

   Fixing solution, 1x, 100 ml:

   Make a 1:10 dilution of the concentrated fixing solution by adding 10 ml of the fixing solution to 90 ml of distilled, deionized water.

   * **Note:** The citrate buffer is designed to be a 0.2M stock solution and can be made in any convenient volume. Check the pH of the stock before use.

2. Wash the membrane to be enhanced in distilled, deionized water. Two rapid 1 minute rinses are sufficient.
3. Discard the water and add the 0.2M citrate buffer. Incubate the membrane in the citrate buffer for approximately 5 minutes. Incubations longer than 5 minutes will not affect the assay. During this step, prepare the enhancement solution.
4. Decant the citrate buffer and add the enhancement solution. Develop the membrane for 2–5 minutes in a location that is protected from light. (Use a dark room, an aluminum foil covering, or a box to shield the reaction from all light.)
5. Stop the reaction by discarding the enhancement solution. Quickly rinse the membrane with water and add enough fixing solution to completely cover the membrane.
6. After 5 minutes, decant the fix solution, wash the membrane twice with excess distilled, deionized water, and air dry the membrane on filter paper.
7. At this point, the staining procedure is completed. The black color of the stained bands is stable and will not fade. The brittle membrane is best stored by sealing it between two sheets of polyester film.

1. **Protein concentration.** In starting any assay, it is important to test for the sample concentration which will provide optimal results for the experiment. To do this, perform an electrophoretic separation of serial dilutions of the sample being used and transfer these proteins to nitrocellulose membrane. Stain the nitrocellulose membrane with the Enhanced Colloidal Gold Total Protein Detection Kit to determine the optimal sample concentration for the specific application. The Mini-PROTEAN® II cell and Mini Trans-Blot module provide a very convenient electrophoresis and blotting system for rapid performance of this test.

2. **Background Problems—Colloidal Gold Total Protein Stain.**

   a. **Membrane coloration.**

   Wash steps: Background coloration can result from shortened wash steps. The three 20 minute washes in TTBS are the minimum requirement for low background noise. These steps are required to remove residual acrylamide from the membrane surface following electrophoretic transfer. The length of incubation or number of washes can be increased, but should not be decreased. Tween-20 is the required detergent for this application. Do not substitute detergents. Also, the distilled, deionized water wash steps prior to adding the Colloidal Gold Total Protein Stain are critical for removal of all buffer salts. Buffer salts will react with the gold sol causing non-specific precipitation of the reagent. The length of incubation or number of washes can be increased, but should not be decreased. A general rule is that the water wash steps should be repeated until the detergent bubbles from any residual Tween-20 are no longer visible in the vessel.

   Incubation in the Colloidal Gold Total Protein Stain: The length of incubation in the gold sol will have an effect on background staining. Longer (overnight) incubations might increase staining of the individual proteins, but they can also produce background staining of the nitrocellulose membrane. The recommended incubation time for the Colloidal Gold Total Protein Stain is 1–4 hours.

   b. **Non-specific detection of streaks and spots following electrophoretic transfer.** The Colloidal Gold Total Protein Stain, like the silver stain for polyacrylamide gels, is very sensitive in its detection of all proteins bound to nitrocellulose membrane. Because of this, extreme care should be taken in all electrophoretic and blotting steps to avoid introducing any extraneous proteins into the system. For gel electrophoresis, these steps include thorough cleaning of all glassware, filtering all solutions (*i.e.* acrylamide preparations, running buffers, and sample buffers) throu nitrocellulose filters, and wearing gloves while working with all components of the system. Gloves are important because skin keratin proteins have been shown to produce contaminating bands between 55–60,000 daltons in SDS-PAGE gels.[4] For electrophoretic transfer, care

must be taken to eliminate nonspecific binding of proteins to the membrane. Nonspecific binding will occur when proteins, which are circulating in the transfer buffer, are indiscriminately driven onto the nitrocellulose membrane. Steps that can be taken to prevent unwanted proteins from entering the blotting system include filtering the transfer buffer to remove protein contaminants; wearing gloves while working with all blotting equipment and reagents; rinsing the fiber pads thoroughly, first in hot water and then in distilled water, following each application; and using clean filter paper. Another potential source of unwanted proteins is from the gel itself. After the nitrocellulose membrane reaches its saturation capacity for the proteins being transferred, the excess molecules can continue through the membrane into the transfer buffer. To prevent these molecules from returning to the membrane as background, a second sheet of nitrocellulose can be inserted into the gel holder cassette prior to electrophoretic transfer. This membrane can be placed before the gel to catch all incoming proteins, or after the first sheet of nitrocellulose to catch proteins that transfer completely through the membrane.

3. **Background Problems—Gold Enhancement Kit**
    a. **Non-specific silver precipitation.** Silver will precipitate in the presence of chloride ions and on exposure to light. Use distilled, deionized water to eliminate contaminants. Perform all reactions in an area protected from direct light. Occasional exposure to light while monitoring the reaction will not affect the overall results.
    b. **Non-specific binding of the Colloidal Gold Total Protein Stain.** Binding of the colloidal gold to the membrane during incubation with the Colloidal Gold Total Protein Stain will increase the background staining with the enhancement step. To reduce this background staining, do not incubate the membrane in the gold sol for more than 4 hours.
    c. **Over development.** The high concentration of gold on the membrane surface causes rapid development of the enhancement reaction. This rapid development will cause non-specific precipitation of the silver reagent during any extended enhancement procedures. Check color formation at 1–2 minute intervals to determine when optimal enhancement has occurred. Optimal results should be obtained in less than 10 minutes.
4. **Reagent Purity.** Use only distilled, deionized water and analytical grade chemicals in making all buffers and stock solutions. Deionized water of less than 1 µmho conductivity is recommended for all steps, as contaminants such as chloride ions will cause non-specific precipitation of the gold sol and gold enhancement reagents.
5. **Temperature.** All steps are designed to be performed at room temperature (22–25 °C).
6. **Reagents.**
    a. **Colloidal Gold Total Protein Stain.** This reagent is designed to be used at full strength. At full strength, the reagent will give rapid staining of most proteins in 30 minutes to 2 hours. Dilution of this reagent will result in decreased performance. The Colloidal Gold Total Protein Stain is naturally red, but will turn dark blue as the gold sol is complexed by freezing or by salt contamination. Be sure to store the gold sol at 4 °C and to avoid contamination of the reagent.

b. **Silver enhancement solution.** This solution is very sensitive to light. Prepare it just before performing the assay to reduce background.

c. **Fixing solution.** The fixing solution scavenges unreacted silver from the membrane surface. This solution can be used to reduce background, but prolonged fixing will decrease sensitivity and should be avoided.

7. **Washes.** 0.3% Tween-20 is essential in the TTBS wash solution to minimize overall background. Other detergents should not be substituted. All wash steps are essential and should not be altered.

8. **Quantitation.** Quantitation of the detected proteins and comparison of protein patterns from different samples can easily be performed using Bio-Rad's Model 620 CCD Densitometer in the reflectance mode.

9. **Technical Service.** Contact Bio-Rad's Technical Service Group in Hercules, California, toll free at 1-800-4BIORAD, or contact your local Bio-Rad representative, if you require any assistance.

Section 4
Equipment and Reagents

4.1 Related Instruments

Catalog Number	Product Description
	Modular Mini Electrophoresis System
165-2940	**Mini-PROTEAN II Cell,** 10 Well Combs, 0.75 mm spacers, includes inner cooling core with gaskets, lower buffer chamber, lid with power cables, 3 sets glass plates, 2 clamp assemblies, two 10 well combs, four 0.75 mm spacers, casting stand with gaskets, leveling bubble, and instructions
170-3930	**Mini Trans-Blot Electrophoretic Transfer Cell,** includes 2 gel holders, buffer chamber with lid and power cables, electrode module, fiber pads, Bio-Ice® cooling unit, and instructions
170-3935	**Mini Trans-Blot Electrode Module** (for use with the Mini-PROTEAN II cell), includes 2 gel holders, electrode module, fiber pads, Bio-Ice cooling unit, and instructions
	Blotting Equipment
170-3910	**Trans-Blot Cell with Wire Electrodes**
170-3939	**Trans-Blot Cell with Plate Electrodes,** complete
170-3946	**Trans-Blot Cell with Plate Electrodes**
170-3940	**Trans-Blot SD Semi-Dry Transfer Cell**
170-6545	**Bio-Dot Apparatus**
170-6547	**Bio-Dot Module**
170-6542	**Bio-Dot SF Apparatus**
170-6543	**Bio-Dot SF Module**
170-3938	**Bio-Dot Microfiltration System,** includes 170-6545 and 170-6543
170-4017	**Mini-PROTEAN II Multiscreen Apparatus**

Catalog Number	Product Description
	Power Supply
165-4761	**Model 200/2.0 Constant Voltage Power Supply,** 100/120 V
165-4762	**Model 200/2.0 Constant Voltage Power Supply,** 220/240 V

4.2 Blotting Media

Catalog Number	Product Description
	Nitrocellulose Membrane (0.45 micron)
162-0115	**Roll,** 33 cm x 3 m, 1
162-0113	**Sheets,** 20 x 20 cm, 5
162-0116	**Sheets,** 15 x 15 cm, 10
162-0114	**Sheets,** 15 x 9.2 cm, 10
162-0117	**Sheets,** 9 x 12 cm, 10
162-0145	**Sheets,** 7 x 8.4 cm, 10
162-0148	**Sheets,** 11.5 x 16 cm, 10
162-0149	**Sheets,** 14 x 15 cm, 10
	Nitrocellulose Membrane (0.2 micron)
162-0112	**Roll,** 33 cm x 3 m, 1
162-0146	**Sheets,** 7 x 8.4 cm, 10
162-0147	**Sheets,** 13.5 x 16.5 cm, 10
	PVDF Membrane
162-0180	**Sheets,** 10 x 15 cm, 10
162-0181	**Sheets,** 15 x 15, 10
162-0182	**Sheets,** 20 x 20 cm, 10
162-0184	**Roll,** 24 cm x 3.3 m, 1
162-0185	**Sheets,** 20 x 20 cm, 3

Section 5
References

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3. Danscher, G., *Histochemistry,* **71,** 81 (1981).
4. Ochs, D., *Anal. Biochem.,* **135,** 470–474 (1983).

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