Biotin-Blot® Protein Detection Kit

Instruction Manual

Catalog Number 170-6512

For Technical Service
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Section 1
Introduction

Bio-Rad's Biotin-Blot Protein Detection Kit is optimized for nanogram level detection of total protein bound to nitrocellulose or Zeta-Probe® nylon membranes. The Biotin-Blot method, which routinely detects 30 ng of membrane bound protein, is 10–50 times more sensitive than anionic stains such as Coomassie® blue, amido black, or fast green, none of which can be applied to nylon membranes. Membranes analyzed for total protein with the Biotin-Blot method can be conveniently compared with a duplicate membrane which has been probed with antibodies or other ligands. Antigenic proteins and immune complexes are easily correlated because direct comparisons of duplicate membranes eliminate the problem of evaluating gels which have undergone shrinkage and shape change.

The Biotin-Blot protein detection method is based on the high affinity of avidin for biotin ($K_D \sim 10^{-15}$M),[12-15] and the stable avidin-biotin complex that results. To perform the assay, protein is first bound to a nitrocellulose or nylon membrane by electrophoretic transfer[1–10] or by passive dot blotting.[11] Primary and secondary amine groups of proteins bound to a nitrocellulose or Zeta-Probe nylon membrane are biotinylated with NHS-Biotin. Following a wash to remove excess biotinylating reagent, the membrane is incubated with avidin-HRP. The resultant avidin-biotin complexes are visualized with the HRP substrate color development procedure. Following color development, proteins bound to nitrocellulose or nylon membranes can be seen as purple bands or "dots."

1.1 Materials Provided

Catalog Number	Product Description	Quantity per Package
170-6518	**Avidin-Horseradish Peroxidase Conjugate (Avidin-HRP)**	2 ml
	and	
170-6529	**N-Hydroxysuccinimide Biotinate in dimethylformamide (NHS-Biotin),** 75 mM	4 ml
170-6531	**Tween-20, EIA grade**	100 ml
161-0715	**Tris**	100 g
170-6534	**HRP Color Development Reagent** (containing 4-chloro-1 naphthol)	5 g

The Biotin-Blot Protein Detection Kit contains enough NHS-biotin and Avidin-HRP reagents to produce 2 liters of each working solution. This is sufficient to assay approximately 500 membrane strips (0.6–0.8 x 10–15 cm size) or 25 membrane sheets (15 x 15 cm size).

1.2 Storage and Stability

The components of the Biotin-Blot Protein Detection Kit should be stored as follows:

	Temperature	Shelf Life
Avidin-HRP	4 °C	1 year
NHS-Biotin	-20 °C	1 year
Tween-20	23–25 °C	>1 year
Tris Base	23–25 °C	>1 year
HRP Color Development Reagent*	-20 °C	1 year

* **Note:** The HRP Color Development Reagent should be stored at -20 °C, desiccated, and away from light.

1.3 Required Reagents Not Included

1. Sodium borate, 10-hydrate ($Na_2B_4O_7 \cdot 10\ H_2O$)–ACS reagent grade.
2. Sodium chloride (NaCl)–ACS reagent grade.
3. Methanol–ACS reagent grade.
4. Hydrogen peroxide–30% H_2O_2 aqueous solution, stabilized, 500 ml (J. T. Baker, catalog number 2186).
5. 1-methyl-2-pyrrolidinone (MPO)–ACS reagent grade (for nylon membranes only).

1.4 Solutions

It is advisable to use the following solutions in volumes of approximately 0.5 ml reagent per cm^2 of membrane. Larger volumes may be used for convenience. The solution should be at least 0.25 cm deep to insure that the membrane is completely submerged during incubation. A 14 cm petri dish will hold one 3 x 5 cm rectangle, a 20 x 20 cm dish will hold one 15 x 15 cm sheet.

The solution volumes that follow are recommended for a typical assay of one 15 x15 cm nitrocellulose or nylon sheet assayed in a 20 x 20 cm incubation vessel, which requires 100 ml of solution to obtain the required depth of 0.25 cm. Adjust volumes for the incubation vessel and the number of membranes being stained. It is best to stain only one membrane per incubation vessel. Should it become necessary to use one vessel for more than one membrane, calculate the solution volume based on the membrane surface area to be stained, not the vessel size.

Solutions for Nitrocellulose Membranes

1. **Borate-Tween solution,** 1 x BT, 2L: (0.05 M $Na_2B_4O_7$•10 H_2O, 0.2% Tween-20 pH 9.3)
   Dissolve 38.14 g $Na_2B_4O_7$•10 H_2O in 1.9 L distilled, deionized water (dd H_20). Add 4 ml Tween-20, bring solution volume to 2 L with dd H_20 and mix.
2. **Tris buffered saline,** 1 x TBS, 2 L: (0.02 M Tris, 0.5 M NaCl)
   Add 4.84 g Tris base to 58.44 g NaCl and bring to 1.8 liters with dd H_2O. Adjust to pH 7.5 with HCl. Add dd H_2O to a final volume of 2 L.
3. **Tris Tween-20 solution,** 1 x TTBS, 1 L: **(**0.02 M Tris, 0.5 M NaCl, 0.2% Tween-20)
   Add 2 ml Tween-20 to 1 L of TBS (above).
4. **Avidin-HRP,** 100ml: Add 100 µl of Avidin-HRP to 100 ml of TTBS.
5. **Phosphate buffered saline,** 1 x PBS, 300 ml: (0.01 M phosphate buffer, pH 7.2)
   Add 0.105 g sodium monobasic phosphate (NaH_2PO_4•H_2O), 0.600 g sodium dibasic phosphate heptahydrate (Na_2HPO_4•7 H_2O), and 2.550 g sodium chloride to 250 ml dd H_2O. Adjust to pH 7.2 with HCl and bring to 300 ml with dd H_2O.
6. **HRP color development solution,** 120 ml:
   a. Dissolve 60 mg HRP Color Development Reagent in 20 ml ice cold methanol. Protect from light. Make fresh daily.
   b. Immediately prior to use, add 60 µl of ice cold 30% H_2O_2 (hydrogen peroxide, stabilized) to 100 ml room temperature TBS. Mix this with (a) above. Use immediately. This will produce a 0.015% H_2O_2 development solution.

Solutions for Nylon Membranes

1. **Borate-Tween,** 1 x BT, 2 L: (0.05 M $Na_2B_4O_7$•10 H_2O, 0.5 M NaCl, 5.0% MPO, 0.2% Tween-20, pH 9.3)
   Dissolve 38.14 g $NaH_2B_4O_7$•10 H_2O and 58.44 g NaCl in 1.9 L dd H_2O. Add 100 ml MPO and 4 ml Tween-20, then bring solution volume to 2 L with distilled, deionized water (dd H_2O) and mix.
2. **Tris buffered saline,** 1 x TBS, 2 L: (0.02 M Tris, 0.5 M NaCl)
   Add 4.84 g Tris base to 58.44 g NaCl and bring to 1.8 liters with dd H_2O. Adjust to pH 7.5 with HCl. Add dd H_2O to a final volume of 2 L.
3. **Tris-Tween-20 solution,** 1 x TTBS, 1 L: (0.02 M Tris, 0.5 M NaCl, 0.2% Tween-20, 5.0% MPO)
   Add 2 ml Tween-20 and 50 ml MPO to 1 L of TBS (above).
4. **Avidin-HRP solution,** 100 ml: Add 100 µl of Avidin-HRP to 100 ml of TTBS.
5. **Phosphate buffered saline,** 1 x PBS, 300 ml: (0.01 M phosphate buffer, pH 7.2)
   Add 0.105 g sodium monobasic phosphate (NaH_2PO_4•H_2O), 0.600 g sodium dibasic phosphate heptahydrate (Na_2HPO_4•7 H_2O), and 2.550 g sodium chloride to 250 ml dd H_2O. Adjust to pH 7.2 with HCl and bring to 300 ml with dd H_2O.
6. **HRP color development solution,** 120 ml:
   a. Dissolve 60 mg HRP Color Development Reagent in 20 ml ice cold methanol. Protect from light. Make fresh daily.
   b. Immediately prior to use, add 60 µl of ice cold 30% H_2O_2 (hydrogen peroxide, stabilized) to 100 ml room temperature TBS. Mix this with (a) above. Use immediately. This will produce a 0.015% H_2O_2 development solution.

Section 2
Total Protein Detection

2.1 General Recommendations

1. **Handling the membrane.** Wear clean plastic gloves or use clean forceps to avoid fingerprints on the membrane. Gloves or forceps which have been in amine containing solution should not be used (see 3 below).
2. **Temperature.** All steps are performed at room temperature (22–25 °C).
3. **Membrane preparation.** Total protein detection is based on the derivatization of the amino groups of proteins bound to nitrocellulose or nylon membranes. Any amine containing materials, such as traces of Tris, or glycine from transfer buffer, decrease the sensitivity of the assay. If the membrane has been in buffer containing amines, it should be rinsed in BT solution at least 3 times, 10 minutes each, prior to beginning total protein detection.
4. **Incubation vessels**. Incubation vessels made of plastic are preferred, since avidin binds to glass even in the presence of detergents. Siliconized glass is acceptable. All vessels should be clean and free of proteins or other amine containing compounds. Such contaminants may compete with membrane bound proteins for the biotinylating reagent and may affect the sensitivity of the assay.
5. **Membrane incubation.** Agitation with a rotating shaker or reciprocating platform enhances incubation efficiency. If neither is available, hand mixing every few minutes and extended incubation periods will suffice.

2.2 Total Protein Detection Procedure

Before beginning, read through the entire procedure. The following procedure is based on 100 ml of incubation solution, which is sufficient to assay one 15 x 15 cm size membrane in a 20 x 20 cm incubation vessel. This volume should be adjusted for the membrane surface area to be stained and the size of the incubation vessel (see Section 1.4).

1. There are three methods by which proteins may be bound to membranes:
   a. Dot-blotting: Cut the nitrocellulose or nylon membrane to the appropriate size (*e.g.* 0.6–0.8 x 15–20 cm strips, 3 x 5 cm rectangles, or 15 x 15 cm squares). If desired, draw a 1 x 1 cm grid on the membrane with pencil. Wet the membrane by slowly placing it in PBS at a 45° angle. Remove the membrane(s) from PBS and dry on filter paper for 5 minutes. Apply 1 to 2 µl of sample to each square using a Hamilton syringe or a variable pipette. Displace the sample to the tip of the syringe or pipette as a drop and touch it to the surface of the membrane. If the protein sample is very dilute, it is possible to apply successive doses at the same spot, taking care to let spot dry completely before applying the next dose. The membrane should be allowed to dry completely (approximately 5 minutes) before continuing to Step 2.
   b. Electrophoretic blotting: Follow the protocol outlined in the Trans-Blot® instruction manual. If desired, cut the membrane into the appropriate lanes containing resolved, transferred proteins. Place membrane strips in the incubation tray and proceed to Step 2.
   c. Microfiltration blotting: The Biotin-Blot protein detection method can easily be adapted for use with Bio-Dot® microfiltration apparatus (catalog number 170-6550). All applications and washes, except for color development, are carried out in the apparatus.
2. Wash the membrane in 100 ml BT solution for 10 minutes. If the membrane has been in a buffer containing amines, repeat the wash 2 times, 10 minutes each.

3. Discard the wash solution and replace it with 100 ml of fresh BT solution. While agitating the incubation vessel, add 200 µl of NHS-biotin. Incubate the membrane for 15 minutes with constant agitation. **Do not prepare BT solution containing NHS-biotin before use, as the biotin reagent is hydrolyzed in aqueous solution.**

   Note: To prevent hygroscopic accumulation of water in the NHS-biotin reagent, equilibrate the vial to room temperature before use and remove reagent with a sterile syringe.
4. Wash the membrane in 100 ml of BT for 5 minutes. Repeat.
5. Wash the membrane in 100 ml of TTBS for 5 minutes. Repeat.
6. Prepare the Avidin-HRP solution as described in Section 1.4. Reserve a 1.0 ml sample for troubleshooting should a problem arise. Incubate the membrane in the Avidin-HRP solution for 1 hour with agitation.
7. Wash the membrane in 100 ml of TTBS for 5 minutes, Repeat.
8. Wash the membrane in TBS for 5 minutes. Repeat.
9. Prepare the HRP color development solution immediately before use as described in Section 1.4. To prevent undesirable precipitates, solution (b) must be at room temperature before addition of solution (a). Reserve a 2.0 ml sample for troubleshooting should a problem arise. Immerse the nitrocellulose or nylon membrane in the color development solution and begin agitation. Protein concentrations greater than 250 ng will immediately become visible as purple bands or dots. Develop color for 15–30 minutes on nitrocellulose membranes or 3–5 minutes on Zeta-Probe membranes (when working with nylon membranes, extended periods of color development will result in increased background). If a heavy precipitate forms in the color development solution, a fresh solution should be prepared and used immediately.
10. Stop color development by immersing the membrane in distilled water for 10 minutes. Change the water at least once to remove remaining color development solution. Take photographs while the membrane is wet to enhance the purple bands or dots. Acceptable photographs can be produced by using Polaroid Type 108, Polacolor 2 Land film, at f8 and 1 second exposure in the reflectance mode. Film should be developed 1 minute.

    Dry nitrocellulose or nylon membranes between sheets of filter paper. Store between polyester sheets. Protect from light to minimize fading.

Section 3 Troubleshooting

3.1 Tests for Monitoring Reagent Activity

A. Activity test for the color development solution.

Combine 1.0 ml of the color development solution with 10 µl of Avidin-HRP conjugate. If the color does not develop in 5 minutes, either the color development reagent or H_2O_2 is at fault. Add 1 µl of fresh 30% H_2O_2. If color does not develop, the color development reagent is suspect. If color develops, H_2O_2 is at fault.

B. Enzyme activity test for the Avidin-HRP solution.

Combine 1.0 ml of the color development solution (tested in 1 above) and 1.0 ml of the Avidin-HRP solution (1:1,000). If color fails to develop with 5 minutes, the conjugate solution is suspect. Repeat the procedure beginning at Step 6 with a freshly prepared dilution of Avidin-HRP.

C. Activity test for the NHS-Biotin reagent.

1. Reagent required
   a. 0.1 N HCl
   b. 1.0 N HCl
   c. 0.05 M $Fe(NO_3)_3$ in 0.5 N HCl
   d. 0.1 N NaOH
   e. Dimethylformamide (DMF)

2. Prepare five test tubes containing the following solutions:
   a. Solution 1: 1.0 ml of 0.1 N NaOH + 10 µl DMF
   b. Solutions 2–4: 1.0 ml of 0.1 N NaOH + 10 µl NHS-biotin reagent
   c. Solution 5: 1.0 ml of 0.1N HCl + 10 µl NHS-biotin reagent
3. Heat solutions 1–4 to 100 °C for 10 minutes. **Do not heat solution 5.**
4. Add 0.1 ml of 1.0 N HCl and 100 µl of 0.05 M $Fe(NO_3)_3$ in 0.5 N HCl to solutions 1–5. Mix thoroughly.
5. Measure the absorbance of each solution at 500 nm. Subtract the absorbance value of solution 1 from that of solutions 2–5.
6. Solutions 2–4 should be a pale red color. Determine the average absorbance value for solutions 2–4. (The absolute absorbance values may vary depending upon the age and quality of the $Fe(NO_3)_3$.)
7. Solution 5 should be pale yellow to colorless. Presence of a red color indicates hydrolysis of the NHS-biotin reagent. If the absorbance of solution 5 is greater than 10% of the average absorbance of solutions 2–4, the NHS-biotin reagent is suspect.

3.2 Troubleshooting Guide

A. No reaction or weak color development

Probable Cause	Recommended Solution
1. Enzyme inactivated.	
a. Tap water or water deionized by polystyrene resin may inactivate the enzyme.	a. Use distilled, deionized H_2O. Other reagents should be of the highest purity.
b. Azide is a potent inhibitor of the enzyme.	b. Do not use sodium azide in any of the solutions, If a bacteriostat is necessary, use thimerosal (Merthiolate®) at a 0.01% concentration.

Probable Cause	Recommended Solution
2. Color development solution is inactive.	2. Follow activity test procedure for color development reagents (see Section 3.1).
a. Reagent improperly stored. Wetness of a regent or an obvious color change may be symptomatic.	a. Store desiccated at -20 °C and away from light.
b. Light inactivation of color development reagent or H_2O_2.	b. Mix solution out of direct light and use fresh, stabilized H_2O_2.
c. Reagent precipitated out of solution.	c. Mix solution at room temperature See Section 1.4. Methanol can also react with Tween -20 to produce a precipitate. This will not interfere with color development.
3. Avidin-HRP conjugate solution is inactive.	3. Follow activity test for conjugate (see Section 3.1).
a. Avidin-HRP conjugate improperly stored.	a. Store at 0–4 °C or freeze aliquots to avoid repeated freeze-thaw cycles.
4. Inactive NHS-biotin solution.	
a. NHS-biotin reagent improperly stored.	a. Store the NSH-biotin reagent at 20 °C. Equilibrate reagent to room temperature before opening. Use a sterile syringe to remove reagent to prevent contamination and condensation of water inside the vial.
b. NHS-biotin reagent is hydrolyzed.	b. The NHS-biotin reagent hydrolyzed in aqueous solutions. NHS-biotin reagent should be added last to the BT solutions containing the membrane.

Probable Cause	Recommended Solution
5. Little or no protein bound to the membrane.	5. Consult the Trans-Blot instruction manual for proper electrophoretic transfer procedures. Refer to the Bio-Dot instruction manual for the proper microfiltration procedure.
a. Amine containing compounds other than membrane bound proteins competing for reagents.	a. Wash membrane thoroughly in BT prior to stain ing (see Membrane Preparation Section 2.1,3).
b. Amine containing compounds or proteins on the surface of the incubation vessel competing for reagents.	b. Wash incubation vessel thoroughly before use. Siliconize glass vessels to prevent reagents from binding to the surface.
c. Proteins bound to membrane may have few accessible amine functional groups.	c. Increase amount of protein transferred onto membrane. Some proteins may not stain as intensely as others.
B. High background	
1. Insufficient washing after avidin-HRP incubation.	1. Increase the number of washes with TTBS from 2 to 3 (see Section 2.2,7).
2. Avidin-HRP concentration too high.	2. Use avidin-HRP reagent recommended 1:1000 dilution. Lower dilution can lead to background development with no increase in sensitivity.
3. Membrane left in color development too long.	3. Remove membrane from color development solution when background begins to develop and rinse in distilled water.

Probable Cause	Recommended Solution
4. MPO omitted from BT and TTBS solutions for nylon membranes.	4. When using Zeta-Probe or other nylon membranes, prepare BT and TTBS solutions according to the instructions for nylon membranes only.

Section 4 References

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