Biotinylated Standards Kit

Catalog Numbers
161-0307
161-0308
161-0312
161-0313
161-0321
161-0322

BIO-RAD

Table of Contents

Section 1
Introduction

Bio-Rad's biotinylated standards are biotinylated proteins that can be used for accurate molecular weight determinations of immune blotted proteins.[1] The biotinylated standards are visualized on blotted membranes next to the sample proteins. The standards are detected in the course of the standard immune blot protocols that are performed to detect sample proteins. The biotinylation does not significantly alter the molecular weights of the standards. Because the sample proteins and the standards are on the same membrane and exposed to the same reagents during detection, accurate molecular weight markers are visualized alongside the sample proteins without increasing the number of steps or length of incubations.

Avidin conjugates bind specifically to biotinylated proteins. Avidin is available as Avidin-Alkaline Phosphatase Conjugate or Avidin-Horseradish Peroxidase Conjugate. The avidin conjugates are detected by using the appropriate color development reagent. Avidin-AP is detected with BCIP and NBT. Avidin-HRP is detected with 4-chloro-1-naphthol.

The biotinylated standards are run with the sample proteins on an SDS-polyacrylamide gel, and electrophoretically transferred to nitrocellulose membrane. The standards are detected by adding the avidin conjugates to the second antibody solution. The appropriate color development reagent is used to detect the standards and sample proteins simultaneously.

The biotinylated standards are available in high, low, and broad molecular weight ranges, and can easily be used in conjunction with Immun-Blot® assay kits from Bio-Rad.

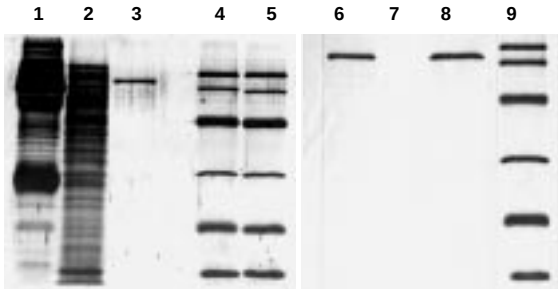

Fig. 1. 12% SDS-polyacrylamide gels run by the method of Laemmli in the Mini-PROTEAN® II cell, and transferred to nitrocellulose in the Mini Trans-Blot® cell. Both gels had the same loading pattern: Lanes 1 and 6, total human serum; Lanes 2 and 7, *E. coli* lysate; Lanes 3 and 8, human transferrin; Lanes 4 and 9, Biotinylated SDS-PAGE Standards, Low Range; Lane 5, SDS-PAGE Molecular Weight Standards, Low Range.

A) Blot was stained with Bio-Rad's Colloidal Gold Total Protein Stain. The biotinylated standards in lane 4 show minimal mobility changes compared to the untreated standards in lane 5.

B) Blot was treated with rabbit anti-human transferrin as the primary antibody. The second antibody solution contained Avidin-HRP and GAR-HRP. The color reaction was done using HRP Color Development Reagent. The blot shows the human transferrin band was positively identified in lanes 6 and 8. The other proteins were not detected by the Avidin-HRP, showing that Avidin-HRP binds specifically to the biotinylated standards.

Section 2
Specifications

Table 1 Composition of the Biotinylated SDS-PAGE Standards

Protein	Source	MW (dalton)	High	Low	Broad	Reference
Myosin	rabbit skeletal muscle	200,000	x		x	2
ß-galactosidase	*E. coli*	116,250	x		x	3
Phosphorylase B	rabbit muscle	97,400	x	x	x	4
Serum albumin	bovine	66,200	x	x	x	5
Ovalbumin	hen egg white	45,000	x	x	x	6
Carbonic anhydrase	bovine	31,000		x	x	7
Trypsin inhibitor	soybean	21,500		x	x	8
Lysozyme	hen egg white	14,400		x	x	9
Aprotinin	bovine pancreas	6,500			x	10

Kit Contents

	Biotin. Stds. Low Range*	Biotin. Stds. High Range*	Biotin. Stds. Broad Range	Avidin-HRP†	Avidin-AP††
161-0307	•••			•••	
161-0308	•••				•••
161-0312		•••		•••	
161-0313		•••			•••
161-0321			•••	•••	
161-0322			•••		•••

* The biotinylated standards contain approximately 130 µg total protein in 50% glycerol, 150 mM NaCl, 100 mM DTT, 3 mM NaN_3.

† Avidin-horseradish peroxidase conjugate is supplied in 10 mM $NaPO_4$, 150 mM NaCl, pH 7.2, containing 1.0% BSA as a stabilizer and 0.01% Thimerosal as a bacteriostat.

†† Avidin-alkaline phosphatase is supplied in 10 mM Tris pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, containing 1.0% BSA as a stabilizer and 0.1% sodium azide as a bacteriostat.

Volume	Biotinylated SDS-PAGE Standards: 250 µl Avidin-HRP: 2 ml Avidin-AP: 1 ml
Applications per kit	Biotinylated SDS-PAGE Standards: Dilute 1:4 for HRP color development, 60-100 applications. Dilute 1:20 for AP color development, 300-500 applications. Avidin-HRP: makes 6 liters, enough for 60 to 120 uses. Avidin-AP: makes 3 liters, enough for 30 to 60 uses.
Storage	Biotinylated Standards: -20 °C Avidin-HRP and Avidin-AP: The conjugates are shipped on dry ice, and can be stored at -20 °C prior to opening. Once thawed, store reagents at 4 °C. Repeated freeze-thaw cycles will damage the reagents.
Shelf life	1 year, if stored correctly.

Note: Biotinylated standards may show extraneous bands when stained with a total protein stain.

Section 3
Safety Instructions

Read the entire instruction manual before beginning the assay.

1. Wear gloves and protective clothing, such as a laboratory coat and goggles, when preparing and working with the solutions in the assay. DMF, 4-chloro-1-naphthol, and BCIP can cause skin and eye irritation, and contact should be avoided. In case of contact, immediately flush the skin and eyes with copious amounts of water for at least 15 minutes, and remove contaminated clothing.
2. Work in well-ventilated areas. Avoid inhalation of vapors when handling solutions containing DMF, 4-chloro-1-naphthol and BCIP.
3. Do not mouth-pipet any solutions.

Section 4
Solutions

4.1 Solutions for Electrophoresis Gels

1. **Sample buffer** (SDS-PAGE reducing buffer)

Distilled water	4.0 ml
0.5 M Tris-HCl pH 6.8	1.0 ml
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml
ß-mercaptoethanol	0.4 ml
0.1% (w/v) bromophenol blue	0.2 ml
	8.0 ml

4.2 Solutions for Nitrocellulose Membranes

1. **TBS** - Tris buffered saline, 2 liters

   20 mM Tris, 500 mM NaCl

   Add 4.84 g Tris base, 58.44 g NaCl to 1.8 liters dd H_2O. Adjust to pH 7.5 with HCl. Add dd H_2O to a final volume of 2 liters.
2. **TTBS** - Tween-20, Tris buffered saline.

   20 mM Tris, 500 mM NaCl, 0.05% Tween-20. Add 0.5 ml Tween-20 to 1 liter of TBS.
3. **Blocking solution,** 100 ml:

   3% gelatin in TBS. Add 3 g gelatin to 100 ml of TBS. Heat to 50 °C with stirring to dissolve, then cool. A microwave oven will quickly solubilize the gelatin, but do not heat above 65 °C.
4. **Antibody buffer,** 200 ml:

   1% gelatin in TTBS. Add 2 g gelatin to 200 ml TTBS. Heat to 50 °C with stirring to dissolve. Cool before adding antibody. 100 ml is used for the first antibody solution and 100 ml is used for the conjugate solution.
5. **First antibody solution,** 100 ml:

   Dilute first antibody to appropriate titer in at least 100 ml antibody buffer.

6. **Second antibody,** avidin-HRP or avidin-AP solution, 100 ml:

   Dilute blotting grade second antibody HRP or AP conjugate 1:3,000 by adding 33 µl to 100 ml antibody buffer. Dilute avidin conjugate 1:3,000 by adding 33 µl to the same solution.

7. **HRP color development solution*,** 120 ml:
   (4-chloro-1-naphthol, catalog number 170-6534)

   a. Dissolve 60 mg of HRP color development reagent in 20 ml methanol. Protect from light. Make fresh daily. Label this solution A.

   b. Immediately prior to use, add 60 µl of 30% H_2O_2 (hydrogen peroxide, stabilized) to 100 ml room temperature TBS. Mix this with solution A. Use immediately. Solution B must be at room temperature before addition of solution A to prevent undesirable precipitates.

8. **Ap color development buffer**,** 1 liter:

   100 mM Tris, 1 mM $MgCl_2$, pH 9.5

   Add 12.1 g Tris to 204 µl $MgCl_2$ solution (4.9 M $MgCl_2 \cdot 6H_2O$) in 1 L dd H_2O. Adjust to pH 9.5 with HCl.

9. **AP color development solution**,** 100 ml:
   (BCIP, catalog number 170-6539, and NBT, catalog number 170-6532)

   a. Dissolve 30 mg NBT and 15 mg BCIP in 1 ml DMF. Vortex until solids are well suspended. Add 1 ml AP color development buffer. Vortex until solids dissolve. Label this solution A.

   b. Immediately prior to use, add solution A to 100 ml of room temperature AP color development buffer. Use immediately. The final concentrations should be 0.3 mg/ml NBT, and 0.15 mg/ml BCIP.

*HRP Conjugate Substrate Kit (catalog number 170-6431) simplifies the color development procedure. The kit contains premixed 4-chloro-1-naphthol and hydrogen peroxide solutions, and preweighed 10 x color development buffer, for easy preparation of color development solutions.

**AP Conjugate Substrate Kit (catalog number 170-6432) simplifies the color development procedure. The kit contains premixed BCIP and NBT solutions, and preweighed 10 x color development buffer, for easy preparation of color development solutions.

Section 5
Assay Procedure

5.1 General Recommendations

1. **Solution volume.** The liquid in the incubation vessel should be at least 0.25 cm deep to insure the membrane is completely submerged during incubation. There should be at least 0.5 ml of reagent per cm^2 of membrane. Larger volumes may be used for convenience.
2. **Handling the membrane.** Wear clean plastic gloves or use forceps to avoid leaving fingerprints on the membrane.
3. **Temperature.** All steps are performed at room temperature (22-25 °C).
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.
5. **Membrane incubation.** Agitation with a rotating shaker platform enhances incubation efficiency. If a shaker platform is not available, hand mixing every few minutes and extended incubation periods will suffice.
6. **Detection.** It is best to detect only one membrane per incubation vessel. Should it become necessary to use more than one membrane per incubation vessel, calculate the solution volume based on the membrane surface area, not the vessel size.

5.2 Protocol for SDS-PAGE Electrophoresis

1. When using HRP conjugates, dilute standards 1:4 in sample buffer. When using AP conjugates, dilute the standards 1:20 in sample buffer. Heat for 5 min at 95 °C. Cool and load 10 µl/well for mini-gels with 1.5 mm thick, 10 well combs. Load 10-15 µl/well for full length gels (16-20 cm) with 1.5 mm thick, 15 well combs. Optimal load volumes will vary with well size and gel thickness. Serial dilutions are recommended to determine optimal loading volumes.
2. Perform the SDS-PAGE electrophoresis under the conditions described in the instrument instruction manual.
3. Electrophoretically transfer the proteins to nitrocellulose membrane following the instructions provided in the Trans-Blot® cell, Trans-Blot SD cell or Mini-Trans-Blot cell manual.

5.3 Protocol for Immune Detection

1. Immerse the membrane at a 45° angle into the proper blocking solution. Gently agitate the solution, using a shaker platform, for 30 minutes to 1 hour.
2. Remove the blocking solution and wash the membrane twice in TTBS, 5 minutes with gentle agitation.
3. Remove wash solution, and add the first antibody solution. Incubate for 1 hour. Longer incubation times may increase sensitivity, but may also increase background.
4. Remove the unbound first antibody by washing the membrane twice for 5 minutes in TTBS with gentle agitation.
5. Remove the wash solution,and add the second antibody, avidin-HRP or avidin-AP conjugate solution. This solution should contain the appropriate dilution of blotting grade second antibody conjugate, protein A or protein G conjugate. The avidin conjugate is used in a 1:3,000 dilution. Use approximately 100 ml for full length gels or 50 ml for minigels. Incubate 1 hour with gentle agitation using a shaker platform. Save 1 ml of the conjugate solution for troubleshooting, if necessary.
6. Remove the conjugate solution. Wash the membrane for 5 minutes in TTBS with gentle agitation. Repeat.
7. Wash in 100 ml of TBS for 5 minutes. Repeat.
8. Prepare the appropriate color development solution immediately before use. Immerse the membrane in the development solution. Allow the color development to proceed until all the bands are detected. If no color develops, consult the troubleshooting guide. For color development reactions longer than 45 minutes, cover the incubation tray to prevent fading. Save 1 ml of the color development solution for troubleshooting, if necessary.
9. Stop the development by immersing the membrane in distilled water for 10 minutes. Change the water at least once.

Note: A small amount of antigen or 1 ng of rabbit, mouse, or human IgG dotted on one corner of the membrane will develop color if the immune detection procedure is successful. This is an excellent check on the operation of the assay and will help you to gauge the rate of color development.

Section 6
Troubleshooting Guide

6.1 Activity Test for Reagents

A. Activity test for HRP color development solution.

Combine 1.0 ml of the color development solution with 5 µl of concentrated avidin-HRP conjugate. The color reaction should develop immediately. If color fails to develop within a few minutes, the color development solution is inactive. Make up fresh working solution and repeat the color development assay.

B. Activity test for AP color development solution.

Combine 1.0 ml of the color development solution with 5 µl full strength avidin-AP. The color reaction should develop immediately. If color fails to develop within a few minutes, the color development solution is inactive. Make up fresh solution and repeat the color development assay.

C. Activity test for avidin conjugate solution.

Combine 1.0 ml of the color development solution (tested above) and 1.0 ml of the 1:3,000 dilution of avidin conjugate. A light blue tinge should develop within 15 minutes. If color fails to develop within 25 minutes, the conjugate solution is suspect. Repeat the procedure with a freshly prepared dilution of conjugate.

6.2 No Reaction or Weak Color Development

A.	**Enzyme inactivation**	
	1. Tap water or water deionized by polystyrene resins may inactivate the enzyme.	Use distilled, deionized water. Other reagents should be of the highest purity.
B.	**HRP enzyme is inactive**	
	1. Azide is a potent inhibitor of HRP enzyme.	Do not use sodium azide in any of the solutions. If a bacteriostat is necessary, use Thimerosal (merthiolate) at a 0.01% concentration.

C. Color development solution is inactive

1.	Reagent improperly stored, or has been exposed to light.	Store according to instructions, and check activity using the activity test procedure for color development.

D. HRP Color Development Solution is inactive

1.	Light inactivation of color development reagent of H_2O_2.	Make solution fresh each time. Use stabilized H_2O_2.
2.	Reagent precipitated out of solution.	Mix solution at room temperature. Methanol can react with Tween-20 to produce a precipitate. This will not interfere with color development.

E. Little or no biotinylated standards bound to the membrane

1.	Electrophoretic transfer may be incomplete.	Silver stain gel to check that all the protein is absent. If protein is present in the gel, consult the Trans-Blot cell instruction manual.

6.3 No Color in Standards, Good Color Development in Sample

A. Avidin conjugate is inactive

1.	Avidin conjugate has been improperly stored.	Check activity using the activity test procedure for conjugate. Avoid repeated freeze-thaw cycles by storing the reagent at 4 °C.

6.4 No Color in Sample, Good Color Development in Standards

For problems with immune detection of the samples, consult Bio-Rad's Immun-Blot assay kit instruction manual.

6.5 High Background

1.	Insufficient blocking prior to first antibody incubation.	Increase blocking step to 60 minutes.
2.	Insufficient washing after avidin conjugate incubation.	Increase the number of washes with TTBS from two to three after each antibody incubation step.
3.	Avidin conjugate concentration too high.	Use avidin conjugate at the recommended dilution. Higher concentrations can lead to higher background without increasing sensitivity.
4.	Membrane left in color development solution too long.	Remove membrane from color development solution when background begins to develop and rinse in distilled water.
5.	Primary or secondary antibody concentration too high.	Perform serial dilutions of each antibody to determine the optimum dilution.
6.	Gelatin blocking solution too old.	Use fresh solution.

Section 7
References

1. Della-Penna, D., Christoffersen, R. E. and Bennett, A. B., *Anal. Biochem.,* **152,** 329 (1986).
2. Woods, E. F., *et al., J. Biol. Chem.,* **238,** 2374 (1963).
3. Fowler, A. V. and Zabin, I., *Proc. Natl. Acad. Sci. USA,* **74,** 1507 (1977).
4. Titani, K., *et al., Proc. Natl. Acad. Sci. USA,* Vol. 74, No. 11, p. 4762 (1977).
5. Brown, J. R., *Fed. Proc.,* **34,** 591 (1975).
6. Warner, R. C., "Egg Proteins", in: **The Proteins,** Vol. IIA, p. 435 (Neurath, H. and Bailey, K. eds.), Academic Press, New York (1954).
7. Davis, R. P., "Carbonic Anhydrase", in: **The Enzymes,** Vol. V, p. 545, (Boyer, P.D., ed.) Academic Press, New York (1971).
8. Wu, Y. V. and Scheraga, H. A., *Biochemistry,* **1,** 698 (1962).
9. Jolles, P., *Angew. Chem., Intl. Edit.,* **8,** 227 (1969).
10. Kassell, B., and Laskowski, M., *Biochem. Biophys. Res. Comm.,* **20,** 463 (1965).
11. Johnson, D. A., *et al., Gene Anal. Techn.,* **1,** 3 (1984).

Section 8
Ordering Information

Catalog Number	Product Description
161-0306	**Biotinylated SDS-PAGE Standards, Low Range,** 250 µl
161-0307	**Biotinylated SDS-PAGE Standards Kit, Low Range,** HRP*
161-0308	**Biotinylated SDS-PAGE Standards Kit, Low Range,** AP*
161-0311	**Biotinylated SDS-PAGE Standards, High Range,** 250 µl
161-0312	**Biotinylated SDS-PAGE Standards Kit, High Range,** HRP*
161-0313	**Biotinylated SDS-PAGE Standards Kit, High Range,** AP*
161-0319	**Biotinylated SDS-PAGE Standards, Broad Range,** 250 µl
161-0321	**Biotinylated SDS-PAGE Standards Kit, Broad Range,** HRP*
161-0322	**Biotinylated SDS-PAGE Standards Kit, Broad Range,** AP*
170-6528	**Avidin-HRP,** 2 ml
170-6533	**Avidin-AP,** 1 ml

* Each kit contains 250 ml biotinylated standards, 2 ml Avidin-HRP or 1ml Avidin-AP, and complete instructions.

Horseradish Peroxidase Enzyme Substrates

170-6431	**Horseradish Peroxidase Conjugate Substrate Kit,** contains pre-mixed 4-chloro-1-naphthol, hydrogen peroxide and 10x color development buffer, produces 1 L of color development reagent.
170-6534	**Horseradish Peroxidase Color Development Reagent** (4-chloro-1-naphthol), 5 g
170-6535	**Horseradish Peroxidase Color Development Reagent, DAB** (3,3'-diaminobenzidine), 5 g

Alkaline Phosphatase Enzyme Substrates

170-6432	**Alkaline Phosphatase Conjugate Substrate Kit,** contains pre-mixed BCIP, NBT and 10x color development buffer, produces 1 L of AP color development reagent

Individual Reagents Necessary for Purple Color Development (Order both)

170-6539	**AP Color Development Reagent BCIP** (5-Bromo-4-Chloro-3-Indolyl Phosphate), 300 mg
170-6532	**AP Color Development Reagent NBT** (Nitro Blue Tetrazolium), 600 mg

Related Accessories

Catalog Number	Product Description
	Immun-Blot Assay Kits*
170-6460	**Immun-Blot Assay Kit - Goat Anti-Rabbit AP Conjugate**
170-6461	**Immun-Blot Assay Kit - Goat Anti-Mouse AP Conjugate**
170-6462	**Immun-Blot Assay Kit - Goat Anti-Human AP Conjugate**
170-6463	**Immun-Blot Assay Kit - Goat Anti-Rabbit HRP Conjugate**
170-6464	**Immun-Blot Assay Kit - Goat Anti-Mouse HRP Conjugate**
170-6465	**Immun-Blot Assay Kit - Goat Anti-Human HRP Conjugate**
170-6466	**Immun-Blot Assay Kit - Protein A HRP**
170-6467	**Immun-Blot Assay Kit - Protein G HRP**

* All Immun-Blot Assay Kits contain enough reagents to assay 200 blotted membrane strips.

	Other Blotting Grade Reagent Kits
170-6512	**Biotin-Blot Protein Detection Kit**
170-6517	**Enhanced Colloidal Gold Total Protein Detection Kit**
170-6538	**Gold Enhancement Kit**
	Individual Blotting Grade Reagents
170-6518	**Goat Anti-Rabbit IgG (H+L), Human IgG Adsorbed, AP Conjugate,** 1 ml
170-6520	**Goat Anti-Mouse IgG (H+L), Human IgG Adsorbed, AP Conjugate,** 1 ml
170-6521	**Goat Anti-Human IgG (H+L), Bovine IgG Adsorbed, AP Conjugate,** 1 ml
170-6515	**Goat Anti-Rabbit IgG (H+L), Human IgG Adsorbed, HRP Conjugate,** 2 ml
170-6516	**Goat Anti-Mouse IgG (H+L), Human IgG Adsorbed, HRP Conjugate,** 2 ml
172-1050	**Goat Anti-Human IgG (H+L), Bovine IgG Adsorbed, HRP Conjugate,** 2 ml
170-6522	**Protein A HRP,** 1 ml

Related Accessories (continued)

Catalog Number	Product Description
170-6425	**Protein G HRP,** 1 ml
170-6426	**Protein G Gold,** 2 ml
170-6527	**Colloidal Gold Total Protein Stain,** 500 ml
170-6529	**NHS-Biotin,** 4 ml
170-6408	**Streptavidin,** 1 mg
170-6537	**Gelatin, EIA Grade,** 200 g
170-6531	**Tween-20, EIA Grade,** 100 ml
161-0716	**Tris,** 500 g
	Blotting Standards
161-0305	**Prestained SDS-PAGE Standards, Low Range,** 50 applications
161-0309	**Prestained SDS-PAGE Standards, High Range,** 50 applications
161-0318	**Prestained SDS-PAGE Standards, Broad Range,** 50 applications
161-0324	**Kaleidoscope Prestained Standards,** 500 µl
161-0325	**Kaleidoscope Polypeptide Standards,** 500 µl

Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547

4006050 Rev C