#7071 Store at -20°C

Phototope®-HRP Western Blot Detection System

Anti-rabbit IgG, HRP-linked Antibody

☑ 50 assays

Cell Signaling TECHNOLOGY®

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For Research Use Only. Not For Use In Diagnostic Procedures.

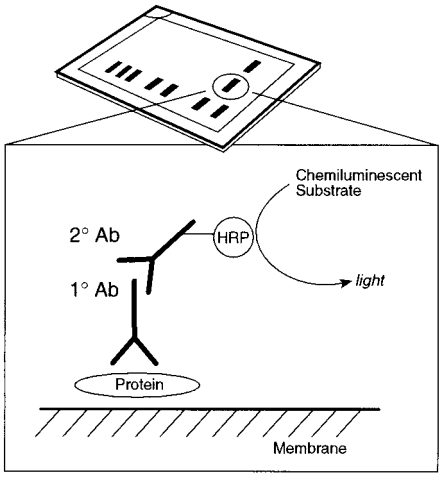
Product Includes	Item #	Kit Quantity	Storage Temp.
Anti-rabbit IgG, HRP-linked Antibody	7074P3	250 µl	-20°C
Anti-biotin, HRP-linked Antibody	7075P4	500 µl	-20°C
Biotinylated Protein Ladder	81851P4	500 µl	-20°C
LumiGLO(R) Reagent A (20X)	95538S	25 ml	4°C
Peroxide Reagent B (20X)	39864S	25 ml	4°C

Storage: Store kit at –20°C. Some kit components may be stored at 4°C as specified on their product labels.

Recommended Antibody Dilutions:

Anti-rabbit IgG, HRP-linked Antibody (#7074)	1:2000
Anti-biotin, HRP-linked Antibody (#7075)	1:1000

Background: Chemiluminescent detection systems have emerged as the best all-around method for detection of western blots. They eliminate the hazards associated with radioactive materials and toxic chromogenic substrates. The speed and sensitivity of these methods are unequalled by traditional alternatives. Because results are generated on film, it is possible to record and store data permanently, and blots detected with chemiluminescent methods are easily stripped for subsequent reprobing with additional antibodies. Horseradish peroxidase (HRP) conjugated secondary antibodies are utilized in conjunction with specific chemiluminescent substrates to generate the light signal. Horseradish peroxidase-antibody conjugates have a very high turnover rate, giving good sensitivity with short reaction times.



After the primary antibody is bound to the target protein, a complex with HRP-linked secondary antibody is formed. The LumiGLO® is added and emits light during enzyme catalyzed decomposition.*

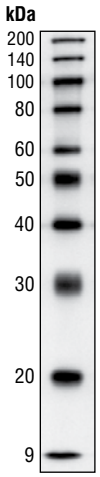
Description: The Phototope®-HRP Western Blot Detection System is designed for the chemiluminescent detection of proteins in standard western blotting applications. Proteins and biotinylated molecular weight markers (provided) are separated by SDS-PAGE and transferred onto membrane. Following incubation with your primary anti-serum, horseradish peroxidase (HRP) linked secondary antibody and HRP-linked anti-biotin antibody are bound and then allowed to react with LumiGLO® reagent. The light emitted by destabilized LumiGLO® reagent is subsequently captured on X-ray film.

Applications: This product has been optimized for use in chemiluminescent western blotting applications.

Method Overview:

There are six basic steps in the western blotting procedures with the Phototope®-HRP Western Blot Detection System.

1. **Polyacrylamide Gel Electrophoresis of Proteins:** Separate the protein samples and molecular weight standards by polyacrylamide gel electrophoresis.
2. **Transfer:** Transfer the protein to membrane by standard electroblotting.
3. **Block Membrane:** Block to saturate nonspecific binding sites on the membrane.
4. **1° Antibody:** Incubate the membrane with the primary antibody.
5. **2° Antibody:** Incubate the membrane with HRP-linked anti-rabbit IgG and HRPlinked anti-biotin antibodies.
6. **Chemiluminescent Detection:** Add LumiGLO® Reagent and capture the emitted light on X-ray film.



11% SDS-PAGE

**LumiGLO® is a trademark of Kirkegaard & Perry Laboratories (KPL). Avoid repeated exposure to skin (see MSDS on our website or request from CST or KPL).*

Phototope® is a trademark of Cell Signaling Technology, Inc.

Western Immunoblotting Protocol (Primary Antibody Incubation in BSA)

For western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

A Solutions and Reagents

NOTE: Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

1. **1X Phosphate Buffered Saline (PBS)**
2. **1X SDS Sample Buffer:** 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red
3. **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
4. **10X Tris Buffered Saline (TBS):** To prepare 1 liter of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X).
5. **Nonfat Dry Milk** (weight to volume [w/v])
6. **Blocking Buffer:** 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk; for 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g nonfat dry milk and mix well. While stirring, add 0.15 ml Tween-20 (100%).
7. **Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T)
8. **Primary Antibody Dilution Buffer:** 1X TBS, 0.1% Tween-20 with 5% BSA; for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g BSA and mix well. While stirring, add 20 µl Tween-20 (100%).
9. **Phototope®-HRP Western Blot Detection System #7071:** Includes Biotinylated Protein Ladder (#81851), Anti-rabbit IgG, HRP-linked Antibody (#7074), Anti-biotin, HRP-linked Antibody (#7075), 20X LumiGLO® Reagent and 20X Peroxide (#7003).

NOTE: #7003 includes LumiGLO® Reagent A (20X) (#95538) and Peroxide Reagent B (20X) (#39864).

10. **Prestained Protein Marker, Broad Range (11-190 kDa) #13953**
11. **Blotting Membrane:** This product has been optimized for nitrocellulose membrane (#12369) which CST recommends. PVDF membranes may also be used.

B Protein Blotting

A general protocol for sample preparation is described below.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.
3. Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
5. Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
6. Microcentrifuge for 5 minutes.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm).

NOTE: CST recommends loading prestained molecular weight marker (#13953, 5 µl/lane) to verify electrotransfer and biotinylated protein ladder (#81851, 10 µl/lane) to determine molecular weights.

8. Electrotransfer to nitrocellulose or PVDF membrane.

C Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) of membrane; for different sized membranes, adjust volumes accordingly.

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
3. Wash three times for 5 minutes each with 15 ml of TBS/T.
4. Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
5. Wash three times for 5 minutes each with 15 ml of TBS/T.
6. Incubate membrane with Anti-mouse IgG, HRP-linked Antibody (#7074) (1:2000) and Anti-biotin, HRP-linked Antibody (#7075) (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature.
7. Wash three times for 5 minutes each with 15 ml of TBS/T.

D Detection of Proteins

1. Prepare substrate by deleting LumiGLO® Reagent A (20X) (#95538) and Peroxide Reagent B (20X) (#39864) to 1X in water (e.g. for 10ml, add 0.5 ml LumiGLO® plus 0.5 ml Peroxide to 9 ml water). Incubate membrane.

NOTE: LumiGLO® substrate can be further diluted if signal response is too fast.

2. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10-second exposure should indicate the proper exposure time.

NOTE: Due to the kinetics of the detection reaction, signal is most intense immediately following LumiGLO® incubation and declines over the following 2 hours.