Applications	Reactivity	Sensitivity	Isotype
W E-P	All	Endogenous	Rabbit

Applications Key: W=Western Blotting E-P=ELISA (Peptide)

Reactivity Key: All=All species expected

Species cross-reactivity is determined by western blot.

Protocols

10001: ELISA Peptide, Western Blotting

ELI ELISA-Peptide Assay Protocol

A. Solutions and Reagents

1. **Carbonate Buffer:** 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.2 g/L NaN_3 (pH 9.6). Use 1 µM synthetic peptide in carbonate buffer.
2. **10X Phosphate Buffered Saline (PBS):** To prepare 1 L add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na_2HPO_4) and 2.4 g potassium phosphate, monobasic (KH_2PO_4) to 1 L dH_2O. Adjust pH to 7.4.
3. **Wash Buffer:** 1X PBS containing 0.05% Tween-20 (PBST)
4. **Blocking Buffer:** 10 mg/ml bovine serum albumin (BSA) in PBST
5. **Antibody Dilution Buffer:** 3% BSA in PBST
6. DELFIA® Europium-labeled Anti-mouse IgG for mouse primary antibodies or Anti-rabbit IgG (PerkinElmer Life Sciences #AD0124) for rabbit primary antibodies.
7. DELFIA® Enhancement Solution (PerkinElmer Life Sciences #1244-105)

(DELFIA® is a registered trademark of PerkinElmer, Inc.)

B. Protocol

1. Coat the wells of a 96-well microtiter plate with 100 µl of 1 µM synthetic peptide in carbonate buffer by incubating overnight at 4 ℃ or for 2 to 6 hours at 37 ℃. If the peptide does not bind or absorb, try other buffers in the pH 4–8 range.
2. Wash plate three times 200 µl/well with wash buffer.
3. Block plate with 200 µl/well blocking buffer for 1 hour at 37°C. Wash plate three times with wash buffer. (May leave dry plate at 4 ℃ for 1–2 months if desired.)
4. Prepare appropriate dilution of primary antibody with antibody dilution buffer. Add 100 µl to wells and incubate at 37°C for 1 hour.
5. Wash three times with wash buffer.
6. Add 67 ng/well DELFIA Europium-labeled Anti-mouse IgG, diluted in 100 µl/well antibody dilution buffer. Incubate at 37 ℃ for 30 minutes.

7. Wash five times with wash buffer.
8. Add 100 µl enhancement solution and incubate at 37°C for 15 minutes. Read plate at 615 nm with an appropriate time-resolved plate reader.

Western Immunoblotting Protocol (Primary Ab Incubation In BSA)

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

Products available from Cell Signaling Technology are linked by their respective catalog numbers.

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

1. **1X Phosphate Buffered Saline (PBS).**
2. **1X SDS Sample Buffer:** (#7722, #7723) 62.5 mM Tris-HCl (pH 6.8 at 25 ℃), 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):** (#9997) 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:** (#9999) (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. **Bovine Serum Albumin (BSA):** (#9998).
9. **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%).
10. **Phototope®-HRP Western Blot Detection System:** (#7071 anti-rabbit) or (#7072 anti-mouse) Includes biotinylated protein ladder, secondary (#7074 anti-rabbit) or (#7076 anti-mouse) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO® chemiluminescent reagent and peroxide.
11. **Prestained Protein Marker, Broad Range (Premixed Format):** (#7720).
12. **Biotinylated Protein Ladder Detection Pack:** (#7727).
13. **Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes, 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 for complete cell lysis and to shear DNA (to reduce sample viscosity).
5. Heat a 20 µl sample to 95–100 ℃ 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 markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 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 ℃.
5. Wash three times for 5 minutes each with 15 ml of TBS/T.

I. For Unconjugated Primary Antibodies

1. Incubate membrane with **appropriate** HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature.
2. Wash three times for 5 minutes each with 15 ml of TBS/T.

II. For HRP Conjugated Primary Antibodies

Skip to Detection of Proteins (Step D).

III. For Biotinylated Primary Antibodies

1. Incubate membrane with HRP-Streptavidin (at the appropriate dilution) in milk for one hour with gentle agitation at room temperature.
2. Wash three times for 5 minutes each with 15 ml of TBS/T.

D. Detection of Proteins

1. Incubate membrane with 10 ml LumiGLO® (0.5 ml 20X LumiGLO®, 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) with gentle agitation for 1 minute at room temperature. **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.

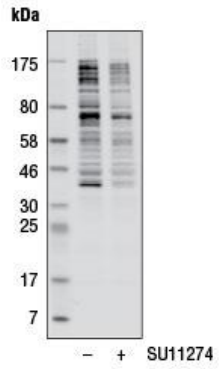
Specificity / Sensitivity

Phospho-Akt Substrate (RXRXXS*/T*) (23C8D2) Rabbit mAb recognizes endogenous proteins containing phospho-Ser/Thr preceded by Arg at positions -5 and -3 in a manner largely independent of the surrounding amino acid sequence. Minor cross-reactivity is observed for proteins that contain phospho-Ser/Thr preceded by Arg at position -3 only. No cross-reactivity is observed with the corresponding nonphosphorylated sequences or with other phospho-Ser/Thr-containing motifs.

Source / Purification

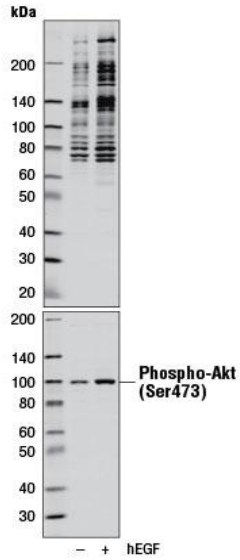
Monoclonal antibody is produced by immunizing animals with an Akt substrate peptide library.

Western Blotting



Western blot analysis of extracts from MKN-45 cells, untreated or treated with c-Met kinase inhibitor SU11274 (1 µM, 2 hr), using Phospho-Akt Substrate (RXRXXS*/T*) (23C8D2) Rabbit mAb. Western blot image was obtained using the Odyssey®Infrared Imaging System (LI-COR®Biotechnology).

Western Blotting



Western blot analysis of extracts from A-431 cells, untreated or treated with Human Epidermal Growth Factor (hEGF) #8916 (10 ng/ml, 20 min), using Phospho-Akt Substrate (RXRXXS*/T*) (23C8D2) Rabbit

mAb (upper), or Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060 (lower). Western blot images were obtained using the Odyssey® Infrared Imaging System (LI-COR® Biotechnology).