| Applications | Reactivity | Sensitivity | Source |
|---|---|---|---|
| W IP E-P | All | Endogenous | Rabbit |

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

**Reactivity Key:** All=All species expected

Species cross-reactivity is determined by western blot.

## Protocols

# 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.

# Immunoprecipitation Protocol / (For Analysis By Western Immunoblotting)

For **shorter assay times** please try our Immunoprecipitation Protocol Utilizing Magnetic Separation / (For Analysis By Western Immunoblotting).

## A. Solutions and Reagents

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

1. 1X Phosphate Buffered Saline (PBS)
2. **1X Cell Lysis Buffer:** (#9803) 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 µg/ml Leupeptin

   **NOTE:** Add 1 mM PMSF immediately prior to use.
3. **Protein A or G Agarose Beads:** (Protein A #9863) Please prepare according to manufacturer's instructions. Use Protein A for rabbit IgG pull down and Protein G for mouse IgG pull down.
4. **3X SDS Sample Buffer:** (#7722) 187.5 mM Tris-HCl (pH 6.8 at 25 ℃), 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue

## B. Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
2. To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 ml ice-cold 1X cell lysis buffer to each plate (10 cm) and incubate the plates on ice for 5 minutes.
4. Scrape cells off the plates and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate samples on ice three times for 5 seconds each.
6. Microcentrifuge for 10 minutes at 14,000 X g, 4 ℃, and transfer the supernatant to a new tube. If necessary, lysate can be stored at –80 ℃.

## C. Immunoprecipitation

**Optional:** It may be necessary to perform a lysate pre-clearing step to reduce non-specific binding to the Protein A/G agarose beads (See section below).

1. Take 200 µl cell lysate and add primary antibody. Incubate with gentle rocking overnight at 4 ℃.
2. Add either protein A or G agarose beads (20 µl of 50% bead slurry). Incubate with gentle rocking for 1–3 hours at 4 ℃.
3. Microcentrifuge for 30 seconds at 4 ℃. Wash pellet five times with 500 µl of 1X cell lysis buffer. Keep on ice during washes.
4. Resuspend the pellet with 20 µl 3X SDS sample buffer. Vortex, then microcentrifuge for 30 seconds.
5. Heat the sample to 95–100 ℃ for 2–5 minutes and microcentrifuge for 1 minute at 14,000 X g.
6. Load the sample (15–30 µl) on SDS-PAGE gel (12–15%).
7. Analyze sample by Western blotting (see Western Immunoblotting Protocol: Western BSA, Western Milk).

## Cell Lysate Pre-Clearing (Optional)

1. Take 200 µl cell lysate and add to either Protein A or G agarose beads (20 µl of 50% bead slurry).
2. Incubate at 4 ℃ for 30 – 60 minutes.

3. Spin for 10 minutes at 4 ℃. Transfer the supernatant to a fresh tube.
4. Proceed to step 1 of Immunoprecipitation.

**NOTE:** For proteins with molecular weights of 50 kDa, we recommend using Mouse Anti-Rabbit IgG (Light-Chain Specific) (L57A3) mAb #3677 or Mouse Anti-Rabbit IgG (Conformation Specific) (L27A9) mAb #3678 as a secondary antibody to minimize masking produced by denatured heavy chains. For proteins with molecular weights of 25 kDa, Mouse Anti-Rabbit IgG (Conformation Specific) (L27A9) mAb #3678 is recommended.

## 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.

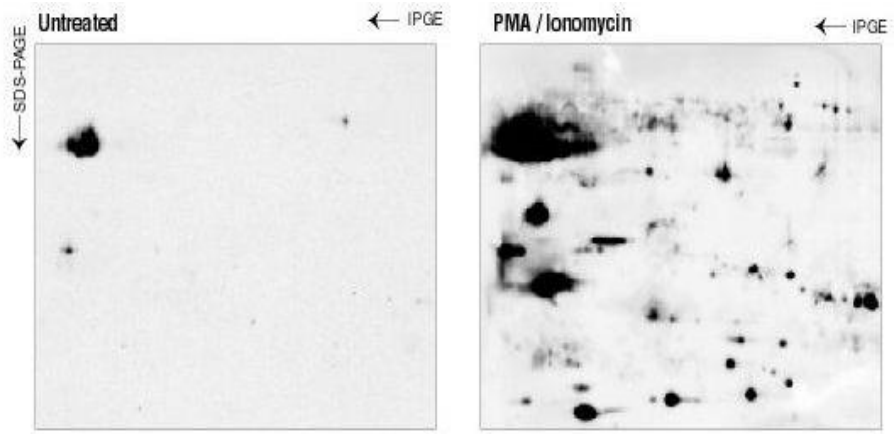
## Specificity / Sensitivity

Phospho-(Ser) PKC Substrate Antibody detects endogenous levels of many cellular proteins only when phosphorylated at serine residues surrounded by Arg or Lys at the -2 and +2 positions and a hydrophobic residue at the +1 position. The antibody does not cross-react with nonphosphorylated serine residues, with phospho-threonine in the same motif, or with phospho-serine in other motifs. (U.S. Patent No's.: 6,441,140; 6,982,318; 7,259,022; 7,344,714; U.S.S.N. 11,484,485; and all foreign equivalents.)

## Source / Purification

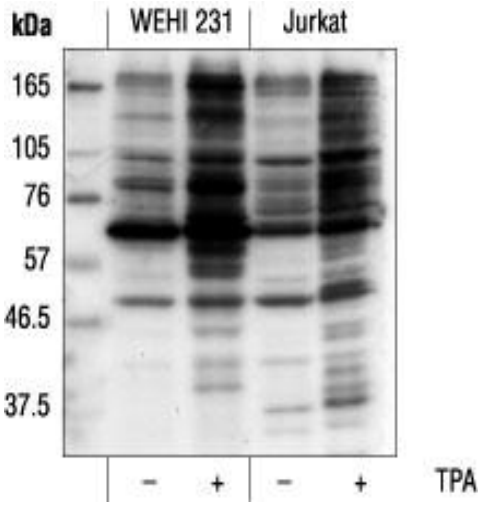
Polyclonal antibodies are produced by immunizing animals with synthetic phospho-PKC substrate peptides . Antibodies are purified by protein A and peptide affinity chromatography.

## Western Blotting



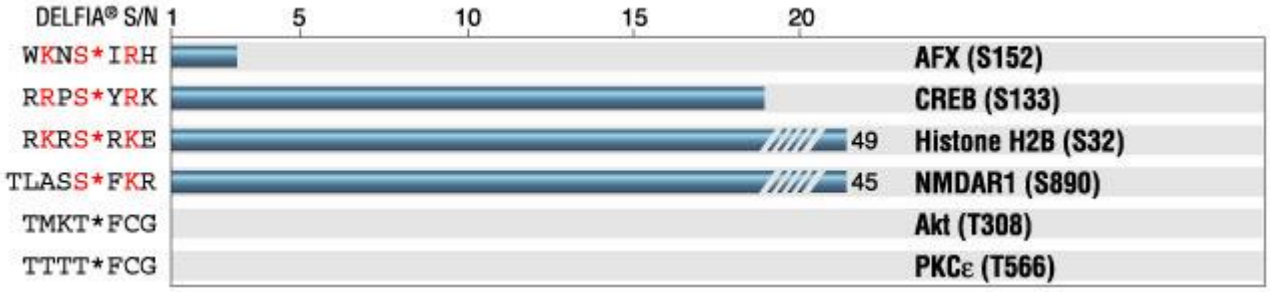

Western blot analysis of extracts from Jurkat cells, untreated or treated with PMA (50 ng/ml) and ionomycin (1 µM for 20 minutes prior to lysis) and subjected to 2-D electrophoresis, using Phospho-(Ser) PKC Substrate Antibody.

## Western Blotting




Western blot analysis of extracts from WEHI 231 cells and Jurkat cells, untreated or TPA-treated, using Phospho-(Ser) PKC Substrate Antibody.

## ELISA-Peptide




Phospho-(Ser) PKC Substrate Antibody ELISA assay: Signal-to-noise ratio of phospho- versus nonphospho-peptides. (S* denotes phosphorylated serine.)