

Phospho-(Ser) Arg-X-Tyr/Phe-X-pSer Motif Antibody

100 µl
(10 Western mini-blot)

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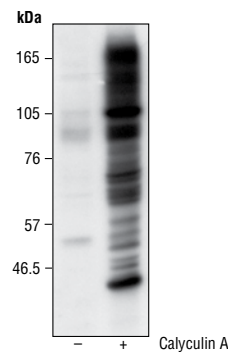
This product is for *in vitro* research use only and is not intended for use in humans or animals.
This product is not intended for use as a therapeutic or in diagnostic procedures.

Applications	Species Cross-Reactivity*	Source	Motif
W, IP, E-P Endogenous	All	Rabbit	RX(Y/F)XS*

Background: The 14-3-3 proteins are a highly conserved family of proteins involved in the regulation of cell survival, apoptosis, proliferation and checkpoint control (1-5). Binding of 14-3-3 is mediated through phospho-serine-containing proteins (6). Two different phospho-serine containing motifs are found using a degenerate phospho-serine-oriented peptide library technique, RSXS*XP and RXY/FXS*XP (6). Motif 2 (RXY/FXS*XP) is found in critical regulatory proteins including cdc25A, cdc25B, PKC γ , IRS-1 and BCR (6). Although Phospho-(Ser) Arg-X-Tyr/Phe-X-pSer Motif Antibody binds 14-3-3 binding motif 2 with no requirement for proline in the +2 position, it provides a powerful tool for the discovery and characterization of potential 14-3-3 binding motif 2-containing proteins or other proteins with the RXY/FXS* motif.

Specificity/Sensitivity: Phospho-(Ser) Arg-X-Tyr/Phe-X-pSer Motif Antibody detects endogenous levels of proteins containing the Arg-X-Tyr/Phe-X-pSer motif. This antibody does not cross-react with nonphosphorylated serine or phospho-threonine proteins with the same motif or other phospho-serine/threonine-containing proteins and peptides without this motif. (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 rabbits with Arg-X-Tyr/Phe-X-pSer-containing peptides. Antibodies are purified by protein A and peptide affinity chromatography.



Western blot analysis of extracts from A431 cells, untreated or calyculin A treated, using Phospho-(Ser) Arg-X-Tyr/Phe-X-pSer Motif Antibody.

Background References:

- (1) Aitken, A. (1995) *Trends Biochem. Sci.* 20, 95–97.
- (2) Zha, J. et al. (1996) *Cell* 87, 619–628.
- (3) Piwnica-Worms, H. (1999) *Nature* 401, 535–537.
- (4) Tzivion, G. et al. (1998) *Nature* 394, 88–92.
- (5) Xing, H. (2000) *EMBO J.* 19, 349–358.
- (6) Yaffe, M.B. et al. (1997) *Cell* 91, 961–971.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at -20°C. Do not aliquot the antibody.

*Species cross-reactivity is determined by Western blot.

Recommended Antibody Dilutions:

Western Blotting 1:1000
Immunoprecipitation 1:20
ELISA-Peptide 1:1000

Companion Products:

Phospho-(Ser) 14-3-3 Binding Motif Antibody #9601
Phospho-(Ser) 14-3-3 Binding Motif (4E2) Mouse mAb #9606
Phospho-Akt Substrate (RXRXS/T) (110B7E) Rabbit mAb #9614
Phospho-(Ser) PKC Substrate Antibody #2261
Phospho-(Thr) MAPK/CDK Substrate Mouse mAb #2321
Phospho-(Ser/Thr) ATM/ATR Substrate Antibody #2851
Phospho-(Ser/Thr) Akt Substrate Antibody #9611
Phospho-(Ser/Thr) PKA Substrate Antibody #9621
Phospho-(Ser/Thr) Phe Antibody #9631
Phospho-(Ser/Thr) Kinase Substrate Antibody Sampler Kit #9920
Phospho-PKA Substrate (RRXS/T) (100G7E) Rabbit mAb #9624
Anti-rabbit IgG, HRP-linked Antibody #7074
Prestained Protein Marker, Broad Range (Premixed Format) #7720
Biotinylated Protein Ladder Detection Pack #7727
20X LumiGLO® Reagent and 20X Peroxide #7003

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IMPORTANT: 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.

Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebra fish B—bovine
Dg—Dog Pg—Pig Sc—S. cerevisiae All—all species expected Species enclosed in parentheses are predicted to react based on 100% sequence homology.

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 Milli-Q or equivalently purified water.

- 1X Phosphate Buffered Saline (PBS)
- 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
- Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
- 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).
- Nonfat Dry Milk (weight to volume [w/v])
- 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%).
- Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T)
- Bovine Serum Albumin (BSA)
- 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%).
- Phototope[®]-HRP Western Blot Detection System #7071:** Includes biotinylated protein ladder, secondary anti-rabbit (#7074) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO[®] chemiluminescent reagent and peroxide.
- Prestained Protein Marker, Broad Range (Premixed Format) #7720
- Biotinylated Protein Ladder Detection Pack #7727
- 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.

- Treat cells by adding fresh media containing regulator for desired time.
- Aspirate media from cultures; wash cells with 1X PBS; aspirate.
- 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.
- Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
- Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
- Microcentrifuge for 5 minutes.
- 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.

- Electrotransfer to nitrocellulose or PVDF membrane.

C Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm²) of membrane; for different sized membranes, adjust volumes accordingly.

- (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
- Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- Incubate membrane with 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.
- Wash three times for 5 minutes each with 15 ml of TBS/T.

D Detection of Proteins

- 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.

- 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.

Immunoprecipitation Protocol / (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:** 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 $\mu\text{g}/\text{ml}$ Leupeptin

NOTE: Add 1 mM PMSF immediately prior to use.

1. **Transfer Buffer:** 25 mM Tris base, 0.2 mM glycine, 20% methanol (pH 8.5)
2. **Protein A or G Agarose Beads:** (Can be stored for 2 weeks at 4°C.) Please prepare according to manufacturer's instructions. Use Protein A for rabbit IgG pull down and Protein G for mouse IgG pull down.
3. **3X SDS Sample Buffer:** 187.5 mM Tris-HCl (pH 6.8 at 25°C), 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°C, and transfer the supernatant to a new tube. If necessary, lysate can be stored at -80°C.

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°C.
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°C.
3. Microcentrifuge for 30 seconds at 4°C. 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°C 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).

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°C for 30 – 60 minutes.
3. Spin for 10 minutes at 4°C. Transfer the supernatant to a fresh tube.
4. Proceed to step 1 of Immunoprecipitation.

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°C or for 2 to 6 hours at 37°C. If the peptide does not bind or absorb, try other buffers in the pH 4–8 range.
2. Wash plate three times 200 $\mu\text{l}/\text{well}$ with wash buffer.
3. Block plate with 200 $\mu\text{l}/\text{well}$ blocking buffer for 1 hour at 37°C. Wash plate three times with wash buffer. (May leave dry plate at 4°C 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 $\mu\text{l}/\text{well}$ antibody dilution buffer. Incubate at 37°C 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.