#2702 Store at –20°C

Zap-70 Antibody

☑ 100 µl
(10 Western mini-blots)

Cell Signaling TECHNOLOGY®

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Applications	Species Cross-Reactivity	Molecular Wt.	Source
W, IP	H, M	70 kDa	Rabbit

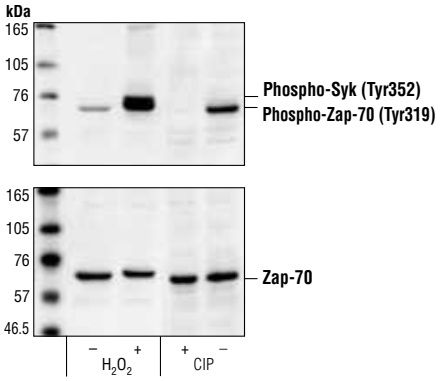
Background: Zap-70, a Syk family protein tyrosine kinase expressed in T and NK cells, plays a critical role in mediating T cell activation in response to T cell receptor (TCR) engagement (1). Following TCR engagement, Zap-70 is rapidly phosphorylated on several tyrosine residues, presumably by two mechanisms: an autophosphorylation and a transphosphorylation by the Src family tyrosine kinase Lck (2-6). Tyrosine phosphorylation of Zap-70 correlates with its increased kinase activity and couples to downstream signaling events.

Specificity/Sensitivity: Zap-70 Antibody detects total levels of endogenous Zap-70.

Source/Purification: Polyclonal antibodies are produced by immunizing rabbits with a synthetic phosphopeptide (KLH coupled) corresponding to residues surrounding the N-terminus of human Zap-70. Antibodies are purified by protein A and peptide affinity chromatography.

Background References:

(1) Chu, D.H. et al. (1998) *Immunol. Rev.* 165, 167–180.
(2) Iwashima, M. et al. (1994) *Science* 263, 1136–1139.
(3) Neumeister, E.N. et al. (1995) *Mol. Cell. Biol.* 15, 3171–3178.
(4) Chan, A.C. et al. (1995) *EMBO J.* 14, 2499–2508.
(5) Williams, B.L. et al. (1999) *EMBO J.* 18, 1832–1844.
(6) Di Bartolo, V. et al. (1999) *J. Biol. Chem.* 274, 6285–6294.



Western blot analysis of extracts from Jurkat cells, starved for 16 hours, and treated with H_2O_2 (2 mM) or with calf intestinal alkaline phosphatase (CIP), using Phospho-Zap-70 (Tyr319) Antibody #2701 (upper) or control Zap-70 Antibody (lower).

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.*

Recommended Antibody Dilutions:

Western blotting	1:1000
Immunoprecipitation	1:100

Companion Products:

Phospho-Zap-70 (Tyr319)/Syk (Tyr352) Antibody #2701

Phototope-HRP Western Blot Detection System, Anti-rabbit IgG, HRP-linked Antibody #7071

Anti-rabbit IgG, HRP-linked Antibody #7074

Prestained Protein Marker, Broad Range (Premixed Format) #7720

Biotinylated Protein Ladder Detection Pack #7727

LumiGLO® Reagent and Peroxide #7003

IMPORTANT: For Western blots, incubate membrane with diluted antibody in 5% BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

Applications Key: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **IC**—Immunocytochemistry **F**—Flow cytometry **E**—ELISA **D**—DELFIA®

Species Cross-Reactivity Key: **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **X**—*Xenopus* **Z**—zebra fish **All**—all species expected
Species enclosed in parentheses are predicted to react based on 100% sequence homology.

Western Immunoblotting Protocol

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

Solutions and Reagents

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

- 1X Phosphate Buffered Saline (PBS)
- 1X SDS Sample Buffer:
  62.5mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50mM DTT, 0.01% w/v bromophenol blue or phenol red
- Transfer Buffer:
  25mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
- 10X Tris Buffered Saline (TBS):
  To prepare 1 liter of 10X TBS: 24.2g Tris base, 80g 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 7.5g 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% blocking buffer; for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0g BSA and mix well. While stirring, add 20 µl Tween-20 (100%).
- Phototope®-HRP Western Blot Detection System #7074:
  Includes biotinylated protein marker, secondary anti-rabbit 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 Marker Detection Pack #7726

Blotting Membrane

This protocol has been optimized for nitrocellulose membranes, which CST recommends. PVDF membranes may also be used.

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^2 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 markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein markers (#7726, 10 µl/lane) to determine molecular weights.

8. Electrotransfer to nitrocellulose membrane.

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 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.
7. Wash three times for 5 minutes each with 15 ml of TBS/T.

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.

Immunoprecipitation / Western Immunoblotting Protocol

Solutions and Reagents

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

- 1X Phosphate Buffered Saline (PBS)
- 1X Cell Lysis Buffer:
  20mM Tris (pH 7.5)
  150mM NaCl
  1mM EDTA
  1mM EGTA
  1% Triton X-100
  2.5mM Sodium pyrophosphate
  1mM β-Glycerolphosphate
  1mM Na_3VO_4
  1µg/ml Leupeptin

Note: CST recommends adding 1mM PMSF before use.*

- Transfer Buffer:
  25mM Tris base, 0.2mM glycine, 20% methanol (pH 8.5)
- Protein A Agarose Beads:
  (Can be stored for 2 weeks at 4°C.) Add 5 ml of 1X PBS to 1.5g of protein A agarose beads. Shake 2 hours at 4°C; spin down. Wash pellet twice with PBS. Resuspend beads in 1 volume of PBS.
- 3X SDS Sample Buffer:
  187.5mM Tris-HCl (pH 6.8 at 25°C), 6% w/v SDS, 30% glycerol, 150mM DTT, 0.03% w/v bromophenol blue

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 1X ice-cold cell lysis buffer plus 1 mM PMSF* to each plate (10 cm^2) and incubate the plate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate four times for 5 seconds each on ice.
6. Microcentrifuge for 10 minutes at 4°C, and transfer the supernatant to a new tube. The supernatant is the cell lysate. If necessary, lysate can be stored at –80°C.

Immunoprecipitation

1. Take 200 µl cell lysate and add primary antibody; incubate with gentle rocking overnight at 4°C.
2. Add protein A 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 by vortexing 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.
6. Load the sample (15–30 µl) on SDS-PAGE gel (12–15%).
7. Analyze sample by Western blotting *(see Western Immunoblotting Protocol)*.