

CD3 ϵ (CD3-12) Rat mAb

100 μ l
(10 Western mini-blot)

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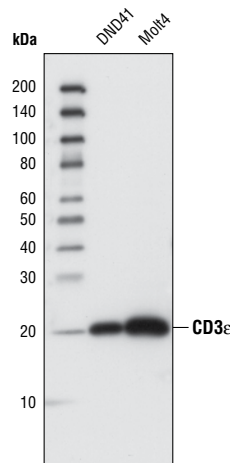
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Applications	Species Cross-Reactivity	Molecular Wt.	Source	Isotype
W, IP, F	H, M	21 kDa	Rat*	IgG1 κ

Background: When T cells encounter antigens via the T cell receptor (TCR), information about the quantity and quality of antigens is relayed to the intracellular signal transduction machinery (1). This activation process depends mainly on CD3 (Cluster of Differentiation 3), a multiunit protein complex that directly associates with the TCR. CD3 is composed of four polypeptides: ζ , γ , ϵ and δ . Each of these polypeptides contains at least one immunoreceptor tyrosine-based activation motif (ITAM) (2). Engagement of TCR complex with foreign antigens induces tyrosine phosphorylation in the ITAM motifs and phosphorylated ITAMs function as docking sites for signaling molecules such as ZAP-70 and p85 subunit of PI-3 kinase (3,4). TCR ligation also induces a conformational change in CD3 ϵ , such that a proline-region is exposed and then associates with the adapter protein Nck (5).

Specificity/Sensitivity: CD3 ϵ (CD3-12) Rat mAb detects endogenous levels of CD3 ϵ protein.

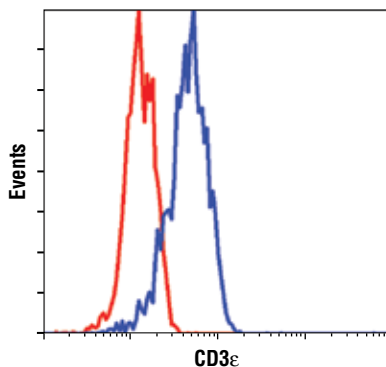
Source/Purification: Monoclonal antibody is produced by immunizing rats with a synthetic peptide corresponding to a region surrounding Pro184 of human CD3 ϵ .



Western blot analysis of total cell lysates from Molt4 and DND41 cells, using CD3 ϵ (CD3-12) Rat mAb.

Background References:

- (1) Kuhns, M.S. et al. (2006) *Immunity* 24, 133–139.
- (2) Pitcher, L.A. and van Oers, N.S. (2003) *Trends Immunol.* 24, 554–560.
- (3) Osman, N. et al. (1996) *Eur. J. Immunol.* 26, 1063–1068.
- (4) Hatada, M.H. et al. (1995) *Nature* 377, 32–38.
- (5) Gil, D. et al. (2002) *Cell* 109, 901–912.



Flow cytometric analysis of Jurkat cells, using CD3 ϵ (CD3-12) Rat mAb Antibody (blue) compared to a nonspecific negative control antibody (red).

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

Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry IC—Immunocytochemistry IF—Immunofluorescence
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken X—Xenopus
Species enclosed in parentheses are predicted to react based on 100% sequence homology.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 μ g/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at -20°C. Do not aliquot the antibody.

***Anti-rat secondary antibodies must be used to detect this antibody**

Recommended Antibody Dilutions:

Western blotting 1:1000
Immunoprecipitation 1:100
Flow Cytometry 1:200

Companion Products:

Zap-70 (99F2) Rabbit mAb #2705
Phospho-Zap-70 (Tyr493) Antibody #2704
Lck Antibody #2752
LAT Antibody #9166
Phospho-LAT (Tyr171) Antibody #3581
Phospho-LAT (Tyr191) Antibody #3584
Anti-rat IgG, HRP-linked Antibody #7077
Prestained Protein Marker, Broad Range (Premixed Format) #7720
Biotinylated Protein Ladder Detection Pack #7727
20X LumiGLO® Reagent and 20X Peroxide #7003

Western Immunoblotting Protocol (Primary Ab Incubation In Milk)

For Western blots, incubate membrane with diluted antibody in 5% w/v nonfat dry milk, 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% nonfat dry milk; for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g nonfat dry milk and mix well. While stirring, add 20 µl Tween-20 (100%).
- Western Blot Detection Reagents:** Anti-rat IgG, HRP-linked Antibody #7077, Biotinylated Protein Ladder Detection Pack #7727, Prestained Protein Marker, Broad Range (Premixed Format) #7720, 20X LumiGLO® Reagent and 20X Peroxide #7003.
- 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.

- 1X Phosphate Buffered Saline (PBS)
- 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: CST recommends adding 1 mM PMSF before use*.

- Transfer Buffer:** 25 mM Tris base, 0.2 mM glycine, 20% methanol (pH 8.5)
- 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.
- 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

- Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
- To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS.

- Remove PBS and add 0.5 ml 1X ice-cold cell lysis buffer plus 1 mM PMSF* to each plate (10 cm) and incubate the plates on ice for 5 minutes.
- Scrape cells off the plates and transfer to microcentrifuge tubes. Keep on ice.
- Sonicate samples on ice four times for 5 seconds each.
- Microcentrifuge for 10 minutes at 4°C, and transfer the supernatant to a new tube. If necessary, lysate can be stored at -80°C.

C Immunoprecipitation

- Take 200 μl cell lysate and add primary antibody. Incubate with gentle rocking overnight at 4°C.
- 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.
- Microcentrifuge for 30 seconds at 4°C. Wash pellet five times with 500 μl of 1X cell lysis buffer. Keep on ice during washes.
- Resuspend the pellet with 20 μl 3X SDS sample buffer. Vortex, then microcentrifuge for 30 seconds.
- Heat the sample to 95–100°C for 2–5 minutes.
- Load the sample (15–30 μl) on SDS-PAGE gel (12–15%).
- Analyze sample by Western blotting (see Western Immunoblotting Protocol).

Flow Cytometry Protocol for Intracellular Staining Using Conjugated Secondary Antibodies

A Solutions and Reagents

- 1X Phosphate Buffered Saline (PBS):** Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 mL distilled water (dH_2O). Adjust the pH to 7.4 with HCl and the volume to 1 liter. Store at room temperature.
- Formaldehyde (methanol free)
- Incubation Buffer:** Dissolve 0.5 g bovine serum albumin (BSA) in 100mL 1X PBS. Store at 4°C

B Fixation

- Collect cells by centrifugation and aspirate supernatant.
- Resuspend cells briefly in 0.5–1 ml PBS. Add formaldehyde to a final concentration of 2–4% formaldehyde.
- Fix for 10 minutes at 37°C.
- Chill tubes on ice for 1 minute.

C Permeabilization

- Permeabilize cells by adding ice-cold 100% methanol slowly to pre-chilled cells, while gently vortexing, to a final concentration of 90% methanol. Alternatively, to remove fix prior to permeabilization, pellet cells by centrifugation and resuspend in 90% methanol.
- Incubate 30 minutes on ice.
- Proceed with staining or store cells at -20°C in 90% methanol.

D Staining Using Unlabeled Primary and Conjugated Secondary Antibodies

NOTE: Allow for isotype matched controls for monoclonal antibodies or species matched IgG for polyclonal antibodies. Count cells using a hemacytometer or alternative method.

- Aliquot 0.5–1x10⁶ cells into each assay tube (by volume).
- Add 2–3 ml Incubation Buffer to each tube and rinse by centrifugation. Repeat.
- Resuspend cells in 100 μl Incubation Buffer per assay tube.
- Block in Incubation Buffer for 10 minutes at room temperature.
- Add the primary antibody at the appropriate dilution to the assay tubes (see individual antibody data sheet for the appropriate dilution).
- Incubate for 30–60 minutes at room temperature.
- Rinse as before in Incubation Buffer by centrifugation.
- Resuspend cells in fluorochrome-conjugated secondary antibody*, diluted in Incubation Buffer according to the manufacturer's recommendations.
- Incubate for 30 minutes at room temperature.
- Rinse as before in Incubation Buffer by centrifugation.
- Resuspend cells in 0.5 ml PBS and analyze on flow cytometer.

*Recommended Secondary Antibodies from Invitrogen.

A-11070 Alexa Fluor® 488 F(ab')₂ fragment of goat anti-rabbit IgG (H+L) (1:1000 dilution)
A-11017 Alexa Fluor® 488 F(ab')₂ fragment of goat anti-mouse IgG (H+L) (1:1000 dilution)