

#2423 Store at -20°C

# Notch4 (L5C5) Mouse mAb

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.

Applications W, IP Transfected	Species Cross-Reactivity* H	Molecular Wt. 80 kDa cleaved 230 kDa full-length	Source Mouse	Isotype IgG1
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**Background:** Notch1 is a transmembrane protein functioning in development and the determination of cell-fate (1). During maturation, the notch molecule is cleaved by a furin-like convertase at its extracellular domain (2). Upon binding to a ligand such as  $\Delta 1$ , or upon extracellular calcium depletion, the carboxy-terminal notch1 fragment is released and further cleaved between Gly1743 and Val1744 (3,4). The resulting activated cytosolic fragment translocates to the nucleus where it activates transcription.

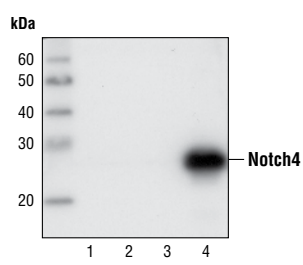
Notch4 is primarily expressed in endothelial cells and is processed similarly to Notch1 (5–7). The mouse *Notch4* gene (also known as *Int-3*) is a frequent target in Mouse Mammary Tumour Virus (MMTV)-induced mammary tumors (6,8). The intracellular domain of Notch4 binds to Smad3 inhibits the TGF- $\beta$  signaling (9).

**Specificity/Sensitivity:** Notch4 (L5C5) Mouse mAb detects levels of the transfected intracellular region of human Notch4 protein.

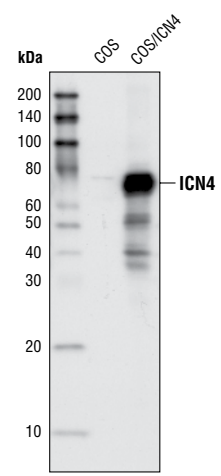
**Source/Purification:** Monoclonal antibody is produced by immunizing mice with a recombinant protein consisting of the Ankyrin repeat domain of human Notch4.

**Background References:**

- (1) Artavanis-Tsakonas, S. et al. (1999) *Science* 284, 770–776.
- (2) Chan, Y.M. and Jan, Y.N. (1998) *Cell* 94, 423–426.
- (3) Schroeter, E.H. et al. (1998) *Nature* 393, 382–386.
- (4) Rand, M.D. et al. (2000) *Mol. Cell. Biol.* 20, 1825–1835.
- (5) Uyttendaele, H. et al. (1996) *Development* 122, 2251–2259.
- (6) Shirayoshi, Y. et al. (1997) *Genes Cells* 2, 213–224.
- (7) Baron, M. (2003) *Semin. Cell Dev. Biol.* 14, 113–119.
- (8) Shirayoshi, Y. et al. (1997) *Genes Cells* 2, 213–224.
- (9) Gallahan, D. and Callahan, R. (1997) *Oncogene* 14, 1883–1890.
- (10) Sun, Y. et al. (2005) *Oncogene* 24, 5365–5374.



Western blot analysis of the recombinant human ankyrin repeat domains of Notch1, 2, 3 and 4 (HANK1-4, lanes 1-4, respectively), using 5 ng of protein per lane and Notch4 (L5C5) Mouse mAb.



Western blot analysis of total cell lysate of COS cells either untransfected or transiently transfected with a construct expressing the intracellular domain of human Notch4 (ICN4), using Notch4 (L5C5) Mouse mAb.

**Entrez-Gene ID** # 4855  
**Swiss-Prot Acc.** # Q99466

**Storage:** Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. 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 1:50

**Companion Products:**

- Cleaved Notch 1 (Val1744) Antibody #2421
- Notch1 (6A5) Rabbit mAb #2495
- MAML1 Antibody #4608
- MAML2 Antibody #4618
- Prestained Protein Marker, Broad Range (Premixed Format) #7720
- Biotinylated Protein Ladder Detection Pack #7727
- 20X LumiGLO® Reagent and 20X Peroxide #7003
- Anti-mouse IgG, HRP-linked Antibody #7076
- Phototope®-HRP Western Blot Detection System, Anti-mouse IgG, HRP-linked Antibody #7072

**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  
 F—Flow cytometry E—ELISA D—DELFIATM  
 Z—zebra fish B—bovine All—all species expected

## Western Immunoblotting Protocol (Primary Antibody 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)
- 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%).
- Phototope®-HRP Western Blot Detection System #7072:** Includes biotinylated protein ladder, secondary anti-mouse (#7076) 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 marker (#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 cm<sup>2</sup>) 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  $\beta$ -glycerophosphate, 1 mM  $\text{Na}_3\text{VO}_4$ , 1  $\mu\text{g/ml}$  Leupeptin

**NOTE:** Add 1 mM PMSF immediately prior to 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 ice-cold 1X cell lysis buffer 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 three times for 5 seconds each.
- 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).

- Take 200  $\mu\text{l}$  cell lysate and add primary antibody. Incubate with gentle rocking overnight at 4°C.
- Add either protein A or G agarose beads (20  $\mu\text{l}$  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  $\mu\text{l}$  of 1X cell lysis buffer. Keep on ice during washes.
- Resuspend the pellet with 20  $\mu\text{l}$  3X SDS sample buffer. Vortex, then microcentrifuge for 30 seconds.
- Heat the sample to 95–100°C for 2–5 minutes and microcentrifuge for 1 minute at 14,000 X g.
- Load the sample (15–30  $\mu\text{l}$ ) on SDS-PAGE gel (12–15%).
- Analyze sample by Western blotting (see Western Immunoblotting Protocol).

### Cell Lysate Pre-Clearing (Optional)

- Take 200  $\mu\text{l}$  cell lysate and add to either Protein A or G agarose beads (20  $\mu\text{l}$  of 50% bead slurry).
- Incubate at 4°C for 30 – 60 minutes.
- Spin for 10 minutes at 4°C. Transfer the supernatant to a fresh tube.
- Proceed to step 1 of Immunoprecipitation.