

#3461 Store at -20°C

Phospho-FLT3 (Tyr591) Antibody

- Small 100 µl (10 Western mini-blot)
- Large 300 µl (30 Western mini-blot)



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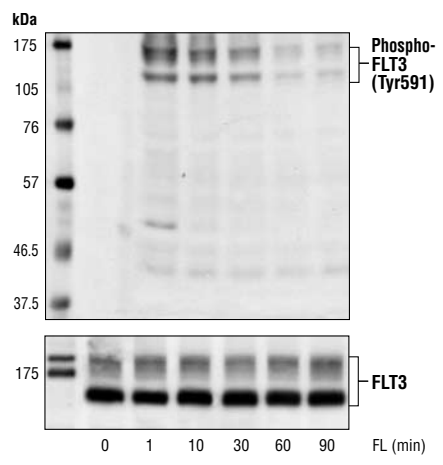
This product is for *in vitro* research use only and is not intended for use in humans or animals. This product is not intended for use as a therapeutic or in diagnostic procedures.

Applications	Species Cross-Reactivity*	Molecular Wt.	Isotype
W, F Endogenous	H, M	130 kDa nonglycosylated form of FLT3 160 kDa glycosylated mature form of FLT3	Rabbit IgG**

Background: FMS-related tyrosine kinase 3 (FLT3, also called Flk2), is a member of the type III receptor tyrosine kinase family, which includes c-Kit, PDGFR and M-CSF receptors. FLT3 is expressed on early hematopoietic progenitor cells and supports growth and differentiation within the hematopoietic system (1,2). FLT3 is activated after binding with its ligand FL, which results in a cascade of tyrosine autophosphorylation and tyrosine phosphorylation of downstream targets (3). The p85 subunit of PI3 kinase, SHP2, GRB2 and Shc are associated with FLT3 after FL stimulation (4-6). Tyr589/591 is located in the juxtamembrane region of FLT3 and may play an important role in regulation of FLT3 tyrosine kinase activity. Somatic mutations of FLT3 consisting of internal tandem duplications (ITDs) occur in 20% of patients with acute myeloid leukemia (7).

Specificity/Sensitivity: Phospho-FLT3 (Tyr591) Antibody detects endogenous FLT3 only when phosphorylated at tyrosine 591. The antibody does not cross-react with other FLT family members. It may cross-react with some tyrosine phosphorylated proteins. (Patent Pending.)

Source/Purification: Polyclonal antibodies are produced by immunizing animals with a synthetic phospho-peptide (KLH-coupled) corresponding to residues surrounding Tyr591 of human FLT3. Antibodies are purified by protein A and peptide affinity chromatography.



Western blot analysis of extracts from BaF3/FLT3 cells, untreated or stimulated with FLT3 Ligand (FL) (3 µg/ml for the indicated times), using Phospho-FLT3 (Tyr591) Antibody (upper) or FLT3 antibody (lower). (130 kDa is the nonglycosylated form of FLT3; 160 kDa is the glycosylated mature form of FLT3.)

Background References:

- Shurin, M.R. et al. (1998) *Cytokine Growth Factor Rev.* 9, 37-48.
- Naoe, T. et al. (2001) *Cancer Chemother. Pharmacol.* 48 Suppl1, S27-S30.
- Namikawa, R. et al. (1996) *Stem Cells* 14, 388-395.
- Beslu, N. et al. (1996) *J. Biol. Chem.* 271, 20075-20081.
- Zhang, S. and Broxmeyer, H.E. (2000) *Biochem. Biophys. Res. Commun.* 277, 195-199.
- Zhang, S. et al. (1999) *J. Leukoc. Biol.* 65, 372-380.
- Mizuki, M. et al. (2000) *Blood* 96, 3907-3914.

Entrez-Gene ID #2322
Swiss-Prot Acc. #P36888

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.

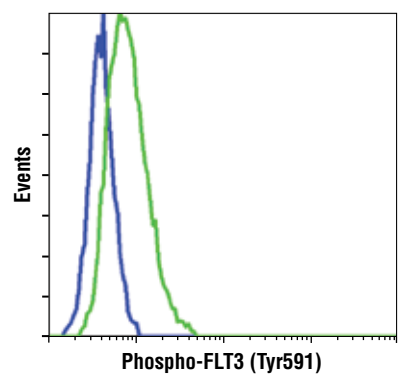
**Anti-rabbit secondary antibodies must be used to detect this antibody

Recommended Antibody Dilutions:

Western Blotting 1:1000
 Flow Cytometry 1:50

Companion Products:

- FLT3 (8F2) Rabbit mAb #3462
- Phospho-FLT3 (Tyr969) (C24D9) Rabbit mAb #3463
- Phospho-FLT3 (Tyr589/591) (30D4) Rabbit mAb #3464
- Phospho-FLT3 (Tyr591) (54H1) Mouse mAb #3466
- Phospho-FLT3 (Tyr591) Antibody (Alexa Fluor® 488 Conjugate) #3459
- Phospho-FLT3 (Tyr591) (33G6) Rabbit mAb #3474
- Phospho-FLT3 (Tyr842) (10A8) Rabbit mAb #4577
- 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
- 20X LumiGLO® Reagent and 20X Peroxide #7003



Flow cytometric analysis of SEM cells, treated with a FLT3 inhibitor (blue) or untreated (green) using Phospho-FLT3 (Tyr591) Antibody.

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.

Flow Cytometry Protocol for Intracellular Staining Using Conjugated Secondary Antibodies

A Solutions and Reagents

1. **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.
2. Formaldehyde (methanol free)
3. **Incubation Buffer:** Dissolve 0.5 g bovine serum albumin (BSA) in 100mL 1X PBS. Store at 4°C

B Fixation

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

C Permeabilization

1. 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.
2. Incubate 30 minutes on ice.
3. 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.

1. Aliquot 0.5-1x10⁶ cells into each assay tube (by volume).
2. Add 2-3 ml Incubation Buffer to each tube and rinse by centrifugation. Repeat.
3. Resuspend cells in 100 μl Incubation Buffer per assay tube.
4. Block in Incubation Buffer for 10 minutes at room temperature.
5. Add the primary antibody at the appropriate dilution to the assay tubes (see individual antibody data sheet for the appropriate dilution).
6. Incubate for 30-60 minutes at room temperature.
7. Rinse as before in Incubation Buffer by centrifugation.
8. Resuspend cells in fluorochrome-conjugated secondary antibody*, diluted in Incubation Buffer according to the manufacturer's recommendations.
9. Incubate for 30 minutes at room temperature.
10. Rinse as before in Incubation Buffer by centrifugation.
11. 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)