Anti-Phospho-MBS (MYPT1) (Thr696) mAb

Cat# CY-M1011

100 µg (1 mg/mL x 100 µL)

Clone Name	Applications	Species Cross-reactivity	Molecular Wt.	Source Isotype
AF20	WB, E, FP	H, M, R, C	135 kDa	Mouse IgG1

Background

The expression of a constitutively active form of Rho-kinase induced stress fiber and focal adhesion formation in fibroblasts and an increase in the level of myosin light chain (MLC20) phosphorylation (1). These results in smooth muscle and in non-muscle cells are attributed to an increase in MLC20 phosphorylation and this is thought to reflect the inhibition of myosin phosphatase (MP). MP is composed of three subunits: a catalytic subunit of type 1 phosphatase delta isoform, PP1c delta, and two non-catalytic subunits, 110 and 20 kDa. The 110 kDa subunit is a targeting molecule and thus has been termed myosin phosphatase target subunit 1 (MYPT1) or myosin-binding subunit (MBS). MBS (MYPT1) is the key molecule involved in regulation of MP activity (2). Phosphorylation by Rho-kinase inhibited MP activity and this reflected a decrease in Vmax. Activity of MP with different substrates also was inhibited by phosphorylation. Two major sites of phosphorylation on chicken MBS (MYPT1) were Thr695 and Thr850. Various point mutations were designed for these phosphorylation sites. Following thiophosphorylation by Rho-kinase and assays of phosphatase activity it was determined that Thr695 was responsible for inhibition.

Rho-kinase, which is activated by GTP-RhoA, phosphorylated MBS (MYPT1) and consequently inactivated myosin phosphatase (MP). Over-expression of RhoA or activated RhoA in NIH 3T3 cells increased phosphorylation of MBS (MYPT1) and MLC20. Thus, Rho appears to inhibit myosin phosphatase through the action of Rho-kinase.

Specificity/Sensitivity: Anti-Phospho-MBS (MYPT1) (Thr696) mAb (AF20) detects endogenous MBS (MYPT1) only when phosphorylated at threonine696. The antibody does not recognize other myosin phosphatase regulatory subunit.

Source/Purification: Monoclonal antibody is produced by immunizing mice with a synthetic phosphopeptide corresponding to residues surrounding Thr696 of human MBS (MYPT1). IgG is purified by protein A-sepharose chromatography.

Recommended Antibody Dilutions: Western blotting: 1-2 µg/mL, ELISA for detection of Rho kinase activity: 1 µg/mL

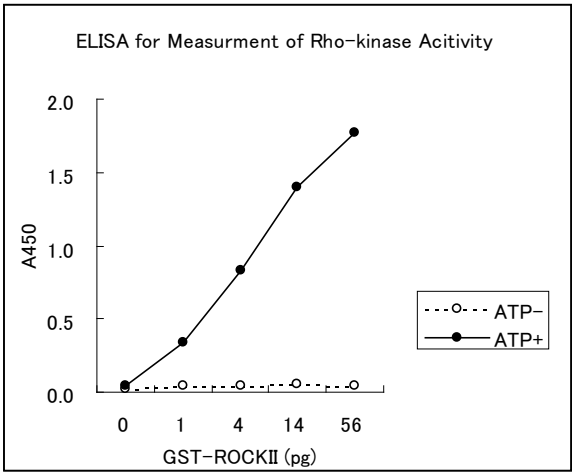
Storage: Supplied in 20 mM phosphate buffer (pH 7.5), 300 mM NaCl, 50 % glycerol. Store at –20°C.

Applications Key: WB: Western Blotting **IP:**Immunoprecipitation **IHC:**Immunohistochemistry **IC:**Immunocytochemistry **F:**Flow cytometry **E:**ELISA **FP:**Fluorescence Polarization assay
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.

References:

1) Kimura, K et al. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 273: 245-248, 1996.
2) Feng J et al. Inhibitory Phosphorylation site for Rho-Kinase on Smooth Muscle Myosin Phosphatase *J.B.C.* **274**, 37385-37390, 1999

Fig.1. ELISA for measurement of recombinant Rho-kinase activity using Anti-Phospho-MBS (MYPT1) (Thr696) mAb (AF20).



Western Immunoblotting Protocol

Solutions and Reagents

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

Transfer Buffer: 25 mM Tris base, 0.2 M glycine, 20 % methanol (pH 8.5)
SDS Sample Buffer (1X): 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
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 %).
10X TBS (Tris-buffered saline): 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).
Primary Antibody Dilution Buffer: 1X TBS, 0.1 % Tween-20 with 5 % blocking agent; 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 %).
Chemiluminescent HRP Detection: secondary anti-mouse antibody conjugated to horseradish peroxidase (HRP), ECL™ chemiluminescent reagent (Amersham Pharmacia)
Wash Buffer TBS/T: 1X TBS, 0.1 % Tween-20
Blotting Membrane: This protocol has been optimized for nitrocellulose membranes, which we recommend. 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 cm2 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).
8. Electrotransfer to nitrocellulose membrane.

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.

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 3 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 3 times for 5 minutes each with 15 mL of TBS/T.
6. Incubate membrane with HRP-conjugated secondary antibody (1:3,000 in 10 mL of Blocking Buffer with gentle agitation for 1 hour at room temperature.
7. Wash 3 times for 5 minutes each with 15 mL of TBS/T.

Detection of Proteins

1. Incubate membrane with 4 mL ECL™ with gentle agitation for 1 minute at room temperature.
2. Drain membrane of excess developing solution, do not let dry, wrap in plastic wrap and expose to x-ray film. An initial ten seconds exposure should indicate the proper exposure time.

Immunoprecipitation Followed by Western Immunoblotting Protocol

Solutions and Reagents

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

Cell Lysis Buffer (1X): 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 Glycerolphosphate, 1 mM Na_3VO_4, 1 µg/mL Leupeptin
Note: We recommend adding 1 mM PMSF before use.
Protein A Agarose Beads: Add 5 mL of 1X PBS to 1.5 g 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. (Can be stored for 2 weeks at 4°C)
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,
Transfer Buffer:25 mM Tris base, 0.2 M glycine, 20 % methanol (pH 8.5)
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 %).

10X TBS (Tris-buffered saline): For 1 liter of 10X TBS, use 24.2 g Tris base and 80 g NaCl. Adjust pH to 7.6 with HCl (use at 1X).
Primary Antibody Dilution Buffer: 1X TBS, 0.05 % 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 10 µL Tween-20 (100 %).
Wash Buffer TBS/T: 1X TBS, 0.1 % Tween-20
Chemiluminescent HRP Detection: secondary anti-mouse antibody conjugated to horseradish peroxidase (HRP), ECL™ chemiluminescent reagent (Amersham Pharmacia)
Wash Buffer TBS/T: 1X TBS, 0.1 % Tween-20
Blotting Membrane: This protocol has been optimized for nitrocellulose membranes, which we recommend. PVDF membranes may also be used.

Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
2. To harvest cells under non-denaturing 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 4 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 2 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.
6. Load the sample (15–30 µL) on SDS-PAGE gel (12–15 %).
7. Analyze sample by Western blotting (see Western Immunoblotting Protocol).

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