Phospho-G-substrate-Thr68/119 Mouse Monoclonal Antibody (Clone 10H11)

Cat# CY-M1017

100 µg (1 mg/ml x 100 µL)

Clone Name	Applications	Species Cross-reactivity	Molecular Wt.	Source Isotype
10H11	WB, ELISA	H, M, R	24 kDa	Mouse IgG2a

Background
G-substrate, an endogenous substrate for cGMP-dependent protein kinase, exists almost exclusively in cerebellar Purkinje cells, where it is possibly involved in the induction of long-term depression. In 1999, Endo et al. identified a G-substrate cDNA by screening expressed sequence tag (EST) databases from a human brain library. The deduced amino acid sequence of human G-substrate contained 2 putative phosphorylation sites (thr68 and thr119) with amino acid sequences that were identical to those reported for rabbit G-substrate. G-substrate mRNA was expressed almost exclusively in the cerebellum as a single transcript. In vitro translation products of the cDNA showed an apparent molecular mass of 24 kD on SDS/PAGE, which was close to that of purified rabbit G-substrate (23 kD). The molecular cloning and expression of recombinant G-substrate should facilitate the investigation of the physiologic role of the protein in cerebellar function.
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Specificity/Sensitivity: Phospho-G-substrate Thr68/119 Antibody detects phosphorylated recombinant G-substrate when phosphorylated at threonine68/119 in vitro by means of western blotting.

Source/Purification: Monoclonal antibody is produced by immunizing mice with a synthetic phosphopeptide corresponding to residues surrounding Thr68 of human G-substrate. IgG is purified by protein A-sepharose chromatography.

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

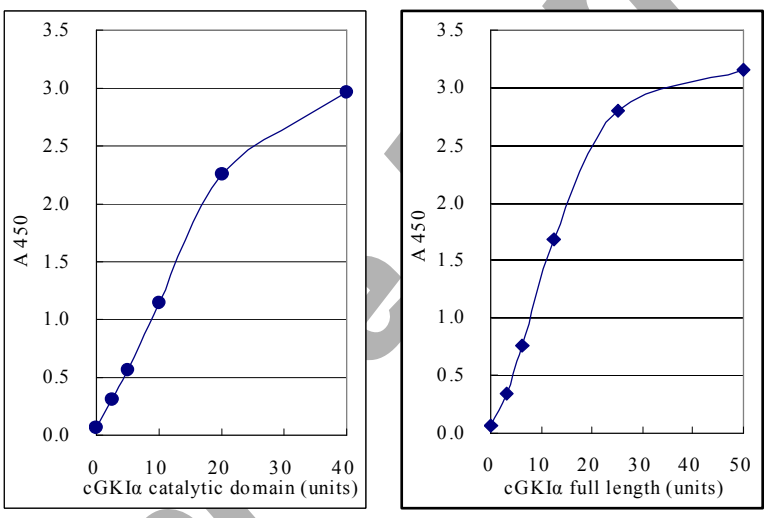
Storage: Supplied in 20 mM phosphate buffer (pH 7.5), 300 mM NaCl, 50 % glycerol. Store at –20°C.

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.

Selected Application References:

1. Endo S, Suzuki M, Sumi M, Nairn AC, Morita R, Yamakawa K, Greengard P, Ito M. Molecular identification of human G-substrate, a possible downstream component of the cGMP-dependent protein kinase cascade in cerebellar Purkinje cells.
   Proc Natl Acad Sci U S A. **96**(5):2467-72, 1999
2. Hall KU, Collins SP, Gamm DM, Massa E, DePaoli-Roach AA, Uhler MD. Phosphorylation-dependent inhibition of protein phosphatase-1 by G-substrate. A Purkinje cell substrate of the cyclic GMP-dependent protein kinase. *J Biol Chem*. **274**(6):3485-95, 1999
3. Aitken A, Bilham T, Cohen P, Aswad D, Greengard P A specific substrate from rabbit cerebellum for guanosine-3':5'-monophosphate-dependent protein kinase. III. Amino acid sequences at the two phosphorylation sites. *J Biol Chem.* **256**(7):3501-6, 1981
4. Schlichter DJ, Detre JA, Aswad DW, Chehrazi B, Greengard P Localization of cyclic GMP-dependent protein kinase and substrate in mammalian cerebellum. *Proc Natl Acad Sci U S A.* **77**(9):5537-41, 1980

Fig.1 ELISA for measurement of recombinant cGK activity using Phospho-G-substrate Thr68/119 Monoclonal antibody (10H11) in CycLex® cyclic GMP dependent protein kinase (cGK) Assay Kit (Cat# CY-1161)



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-rabbit 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 (1:3000 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-rabbit 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 cm2) 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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