

Anti-Phosphotyrosine Mouse MAb

Cat. # APY03-S

Upon arrival, store at 4°C (desiccated)
 See datasheet for storage after reconstitution

Form:	Lyophilized powder
Amount of material:	1 x 25 µl when reconstituted
Validated applications:	WB, IF, IP, ELISA
Species reactivity:	All
Host/Isotype:	Mouse/IgG2b
Clone:	27B10.4

Background Information

Tyrosine phosphorylation, a reversible process, is one of the most frequent post-translational modifications of proteins and is crucial in mediating signal transduction in eukaryotic cells after exposure to cytokines and growth factors (1). Anti-phosphotyrosine antibodies have been important tools in studying the level of tyrosine phosphorylation of proteins in different cellular models. They have also played an important role in enriching phosphotyrosine peptides from trypsin-digested cell lysates. As a result a large number of phosphopeptides have been identified under various physiological and pathological conditions with mass spectrometry technologies (2-3).

Material

APY03 anti-phosphotyrosine antibody is a mouse monoclonal antibody that recognizes proteins post-translationally modified by phosphorylation of tyrosine residues. APY03 was raised against a proprietary mixture of phosphotyrosine peptides conjugated to KLH. It has been shown to recognize a wide range of tyrosine phosphorylated proteins in NIH3T3 cells treated with H₂O₂/vanadate (Figure 1) and can detect 10 ng of phosphotyrosine-labeled bovine serum albumin (see Certificate of Analysis [COA]). APY03 is purified by protein G affinity chromatography and is supplied as a lyophilized white powder. Each Lot of antibody is quality controlled to provide a high batch to batch consistency. The Lot specific µg per tube can be found in the Lot specific COA documents at www.cytoskeleton.com. APY03 shows high specificity to phosphotyrosine peptides and does not cross-react with phosphoserine/threonine peptides in a western blot or an ELISA assay (Figures 1 & 4).

Storage and Reconstitution

Shipped at ambient temperature. The lyophilized protein can be stored desiccated at 4°C for 6 months. For reconstitution, the product tube should be briefly centrifuged to collect the powder at the bottom of the tube.

Reconstitute each tube first in 12.5 µl of Milli-Q water and then add 12.5 µl of glycerol. Alternatively resuspend in 25 µl of 50% glycerol (room temperature). We do not recommend using 50% glycerol at 4°C as this can cause the lyophilized antibody to stick to the pipet tip during resuspension. Store reconstituted antibody at -20°C. Final buffer composition is 200 mM PIPES, 50% glycerol, 1% sucrose, and 1% dextran.

When stored and reconstituted as described, the product is stable for 6 months at -20°C. NOTE: We recommend adding an antibacterial such as sodium azide (0.02% final concentration) to prevent bacterial contamination of the antibody stock.

Applications

Western Blot (WB) Applications

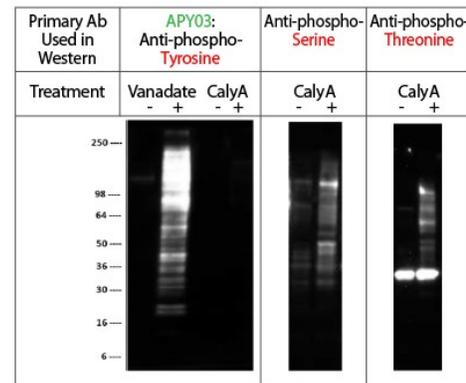
Use as indicated in method at 1:500 dilution, sufficient for 12.5 ml of working strength Ab.

Western Blot Method:

- Run protein samples and control samples on SDS-PAGE.
- Equilibrate the gel in Western blot buffer (25 mM Tris pH 8.3, 192 mM glycine, 5% methanol) for 15 min at room temperature prior to electro-blotting.
- Transfer the protein to a PVDF membrane overnight at 40V at 4°C.
- Wash the membrane once with TBST for 10 minutes (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20).
- Block the membrane surface with 5% nonfat-dry milk in TBST for 60 min at room temperature with constant agitation.
- Incubate the membrane with a 1:500 dilution of APY03 antibody diluted in TBST for 1h at room temperature or overnight at 4°C with constant agitation.

- Wash the membrane 3 times in TBST for 10 min each.
- Incubate the membrane with an appropriate dilution (e.g., 1:20,000) of anti-mouse secondary antibody (e.g., goat anti-mouse HRP conjugated IgG from Jackson Labs., Cat. # 115-035-068) in TBST/3% non-fat milk for 30 min at room temperature.
- Wash the membrane 6 times in TBST for 10 min each.
- Use an enhanced chemiluminescence detection method to detect the signal (e.g., SuperSignal West Dura Extended Duration Substrate; ThermoFisher).

Figure 1: Western Blot: Demonstration of APY03 phosphotyrosine specificity



Legend: A431 cells were either treated (+) or untreated (-) with Calyculin A (CalyA: a serine/threonine phosphatase inhibitor, 50nM for 1 hour). NIH3T3 cells were either treated or untreated with H₂O₂-activated sodium orthovanadate (Vanadate: a specific tyrosine phosphatase inhibitor, 100 µM for 10 minutes). 16 µg of each lysate was resolved in SDS-PAGE and proteins were transferred to PVDF membrane. Membranes were probed as follows:

APY03 primary Ab: Detected tyrosine phosphorylated proteins in Vanadate-treated cells but not serine or threonine phosphorylated proteins in CalyA-treated cells.

Anti-phosphoserine & anti-phosphothreonine primary Ab: Detected serine/threonine phosphorylated proteins in CalyA-treated cells, demonstrating that phosphoserine/threonine proteins are present in CalyA treated lysates but are not detected by APY03. The anti-phosphoserine and anti-phosphothreonine Abs are from Millipore, Cat# AB1603 & AB1607 respectively.

Immunoprecipitation (IP) Applications

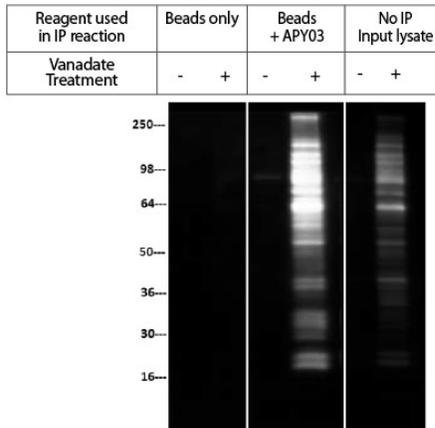
Use as indicated at 5 µl per IP reaction (200-500 µg total lysate per IP), sufficient for approximately 5 IP assays.

IP Method

- Add 5 µl of antibody to 500 µl of PBS pH 7.4 in a microfuge tube containing 30 µl of packed protein G agarose pre-equilibrated in PBS.
- Gently rotate the reaction for 1 hour at 4°C.
- Add 500 µl of PBST to the mixture and centrifuge for 1 minute at 4°C and 3000 rpm (960 x g). Addition of the PBST will prevent agarose from sticking to the microfuge tube walls.
- Discard supernatant and wash beads 3X in PBST (500 µl per wash).
- Add 200-500 µg of cell lysate (0.5 mg/ml protein in RIPA buffer) to the beads.
- Gently rotate the reaction at 4°C for 1 hour.
- Spin down agarose for 1 minute in 4°C at 3000 rpm (960 x g).
- Discard supernatant and wash beads with 800ul of wash buffer (50mM Tris pH7.5, 150mM NaCl and 1% IGEPAL). Spin down agarose for 1 minute in 4°C at 3000 rpm (960 x g).

- Repeat wash two more times. Carefully remove all supernatant without disturbing beads.
- Resuspend beads in 30 μ l of **2X non reducing SDS sample buffer** and incubate at room temperature for 5 minutes.
- Spin down bead for 1 minute at 14,000 rpm (21,000 x g). Carefully remove 25 μ l of supernatant and transfer to a new tube and add 1 μ l of 2-mercaptoethanol. Boil for 5 minutes.
- Load samples on SDS-PAGE for subsequent western blot analysis. NOTE: to avoid detection of Ab heavy and light chains, we recommend using HRP conjugated CleanBlot (Thermo Scientific, #21230) as the secondary antibody reagent.

Figure 2: IP of pervanadate-treated lysates from NIH3T3 cells



Legend: NIH3T3 cells were either treated (+) or untreated (-) with H_2O_2 /orthovanadate (100 μ M for 10min). Cell lysate was prepared in RIPA buffer and 200 μ g of lysate per reaction was used for immunoprecipitation of tyrosine-phosphorylated proteins. APY03 was first bound to protein G beads and then incubated with cell lysate. For bead only control, cell lysate was incubated with protein G beads without APY03. Western blots of immunoprecipitated proteins were developed using APY03 at 1:500 dilution and CleanBlot (Thermo Scientific, #21230) as secondary antibody. No IP input lysate represents the signal from 5% of H_2O_2 /orthovanadate treated or untreated NIH3T3 lysate. As shown in Figure 2, APY03 was able to enrich a wide range of tyrosine-phosphorylated proteins from NIH3T3 cells treated with H_2O_2 -activated orthovanadate. No signal was detected with Protein G bead control without APY03.

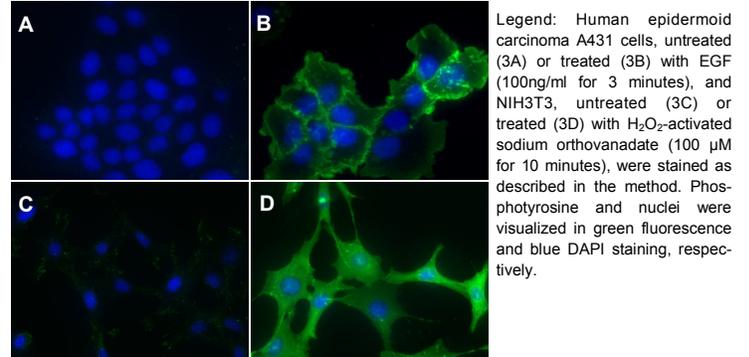
Immunofluorescence (IF) Applications

Use as indicated below at 1:500-1:1000 dilution, sufficient for 12.5-25 ml of working strength Ab, approx. 125-250 IF slides.

IF Method

- Plate cells to required density in suitable growth media on acid washed coverslips in tissue culture dish.
- Allow cells to grow for 24-48 hours then treat one set of coverslips with EGF (100ng/ml for 3 minutes, A431 cells), the other with H_2O_2 -activated sodium orthovanadate (100 μ M for 10 minutes, NIH3T3 cells).
- Fix cells by dipping coverslips in $-20^\circ C$ methanol for 5 minutes (it is convenient to use a porcelain coverslip rack for fixing and rinsing samples).
- Remove coverslips and dip into $-20^\circ C$ acetone for 2 minutes.
- Remove coverslips and air dry coverslips at room temperature.
- Rehydrate cells by dipping coverslips in PBS and then place coverslips, cell side up, on parafilm and apply 100-200 μ l APY03 solution (1:500 in PBS for A431 cells and 1:1000 in PBS for NIH3T3 cells).
- Incubate at room temperature for 60 minutes.
- Wash coverslips three times in PBS at room temperature over a 5 minute period.
- Apply fluorescently labeled anti-mouse secondary antibody at manufacturers recommended dilution. For example, we use Alexa Fluor 555 goat anti-mouse (Life Technologies, Cat# A-21424) at 1:500 dilution in PBS.
- Incubate at room temperature for 60 minutes.
- Wash coverslips three times in PBS at room temperature over a 5 minute period.
- Rinse coverslips briefly in sterile water.
- Dip coverslips in absolute ethanol at room temperature for 2 minutes and transfer to blotting paper, cell side up, and allow to dry.
- Place coverslips, cell side down, on glass slide with mounting media (e.g. EMS, Cat# 17987-10) and observe cells under fluorescence microscope.

Figure 3: IF detection of phosphotyrosinated proteins in vanadate-treated cells



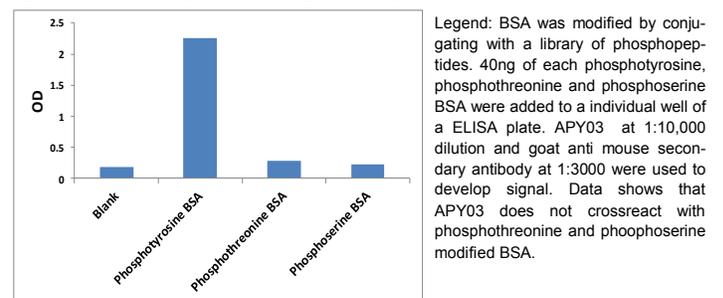
ELISA Applications

Use as indicated below at 1:10,000 dilution, sufficient for approximately 5,000 assays.

ELISA Method

- Add 40 ng in 50 μ l PBS of phosphopeptide BSA to the wells of ELISA plate.
- Incubated plate overnight at $4^\circ C$.
- Wash plate 2 times with 200 μ l PBS.
- Add 200 μ l of 3% milk in PBS and incubate at room temperature for 1 hour.
- Wash plate 3 times with 200 μ l PBS.
- Add 50 μ l of diluted APY03 (1:10,000 dilution in PBS). Incubated at room temperature for 1 hour with shaking.
- Wash plate with 3 times with 200 μ l PBST.
- Add 50 μ l of diluted goat anti-mouse secondary antibody (1:3000 dilution in 3% milk/PBST). Incubate at room temperature for 1 hour with shaking.
- Wash plate 3 times with 200 μ l PBST.
- Add 50 μ l of substrate to each well. Stop reaction by adding 50 μ l of stop solution.
- Measure OD at 490nm.

Figure 4: ELISA assay demonstrating specificity of APY03 for phosphotyrosine



References

- Machida, K. et al. (2003) Profiling the global tyrosine phosphorylation state. Mol. Cell. Proteomics 2, 215-233
- Blagoev, B. et al. (2004) Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. Nat. Biotechnol. 22, 1139-1145
- Schmelzle, K. et al. (2006) Temporal dynamics of tyrosine phosphorylation in insulin signaling. Diabetes 55, 2171-2179

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