V 1.4

Anti-Phosphotyrosine Affinity Beads

Cat. # APY03-Beads

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

Form:	Lyophilized powder
Amount of material:	4 x 330 µl when reconstituted
Validated applications:	IP
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 proteins from whole cell lysates and peptides from trypsin-digested cell lysates. As a result a large number of phosphopeptides have been identified under various physiological and pathological conditions (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. 1 mg of APY03 is covalently conjugated to 1ml of Protein G beads. Each Lot of APY03-Beads conjugation is quality controlled to provide a high batch to batch consistency. The Lot specific µg per µl beads can be found in the Lot specific COA documents at www.cytoskeleton.com. APY03-Beads have been shown to enrich a wide range of tyrosine phosphorylated proteins in HeLa cells treated with pervanadate and A431 cells treated with EGF (Figure 1) and to efficiently enrich specific phosphotyrosine proteins from crude cell lysates (Figure 2).

Storage and Reconstitution

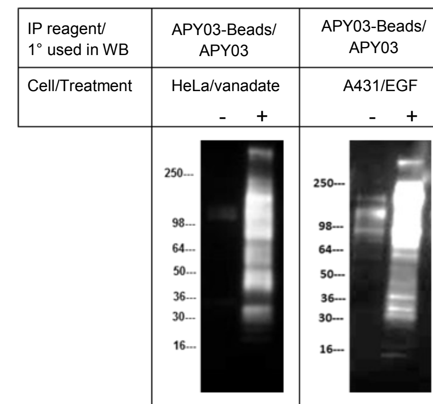
Shipped at ambient temperature. The lyophilized APY03-Beads can be stored desiccated at 4°C for 6 months. For reconstitution, the product tube should be briefly centrifuged to collect the lyophilized beads at the bottom of the tube. Reconstitute each tube in 330ul of Milli-Q water to achieve 50% slurry and store at 4°C. Alternatively, reconstitute in 330 µl of 50% glycerol and store in –20°C. In both cases, allow beads to rehydrate completely before use (15-20 minutes). Final buffer composition is 200 mM PIPES, 5% sucrose, and 1% dextran. When stored and reconstituted as described, the product is stable for at least 6 months in 4°C and 12 months in –20°C.

Applications

Immunoprecipitation (IP) Application

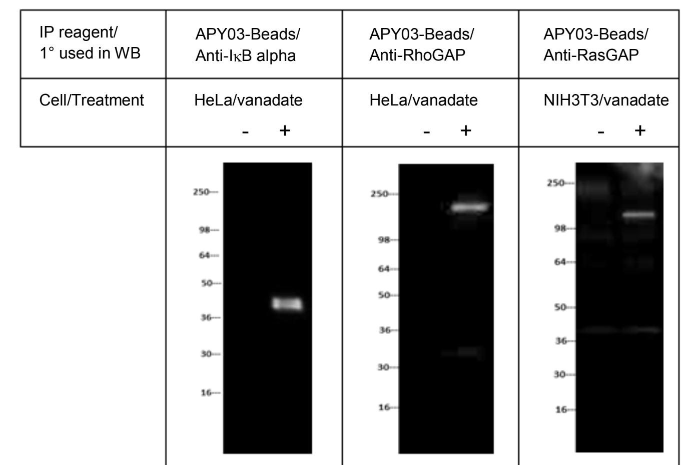
Use 15-30 µl bead slurry for IP. Sufficient for 40-80 IP reactions. See Figures 1 & 2 for representative data.

Figure 1: Enrichment of phosphoproteins from cell lysates



Legend: HeLa cells were either treated or untreated with 100uM of H_2O_2 activated sodium othovanadate for 30 minutes. A431 cells were serum starved for 24 hours before treatment with EGF (50ng/ml for 5 min). Cell lysate was obtained using RIPA buffer. 1 mg of cell lysate was used to incubate with 30µl of APY03-Bead slurry. Eluted proteins were resolved in SDS-PAGE and transferred to PVDF membrane. Anti-phosphotyrosine antibody APY03 (1:500) and goat-anti-mouse secondary (1:20000, Jackson Labs # 115-035-068) were used to detect tyrosine phosphorylated proteins. Western blot was developed with SuperSignal West Dura chemiluminescent reagent (Thermo Scientific) and exposure time was 20 seconds.

Figure 2: Enrichment of IkB alpha, RhoGAP and RasGAP proteins from cell lysates



Legend: Hela cells were either treated or untreated with 100uM of H_2O_2 activated sodium othovanadate for 30 minutes. NIH3T3 cells were either treated or untreated with 100uM of H_2O_2 activated sodium othovanadate for 10 minutes . Cell lysate was obtained using RIPA buffer. 1 mg of cell lysate was used to incubate with 30 µl of APY03-Bead slurry. Eluted proteins were resolved in SDS-PAGE and transferred to PVDF membrane. Primary antibodies, anti-IkB alpha (1:1000, BD Biosciences # 610690), anti-RhoGap (1:1000, Millipore # 05-378) and anti-RasGap (1:1000, BD Biosciences # 610040) and goat-anti-mouse secondary (1:20000, Jackson Labs # 115-035-068) were used in western blot analysis. Western signal was developed with SuperSignal West Dura chemiluminescent reagent (Thermo Scientific) and exposure time for IkB alpha, RhoGAP and RasGAP were 5, 10 and 3 minutes respectively. Figure 2 shows that APY03-Beads are able to immunoprecipitate endogenous tyrosine phosphorylated IkB alpha (~38kDa) and RhoGAP (~190kDa) from Hela cells treated with sodium orthovanadate and tyrosine phosphorylated RasGAP (~120kDa) from NIH3T3 treated with sodium orthovanadate.

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IP and WB Method:

1. Transfer 30 µl of APY03-Bead slurry into an eppendorf tube. NOTE: We recommend cutting the end of the pipette tip by 3-4 mm to generate a wider bore for bead transfer and we have found that gentle treatment of the beads helps eliminate any antibody light/heavy chain signals in the final western blot.
2. Wash the beads 2 times with 1 ml each of PBST. Beads are pelleted by centrifugation (800 x g, 4°C, 1 min) between washes.
3. Incubate the beads with 1 mg of cell lysate (1mg/ml) for 2 hr or overnight night if convenient at 4°C with rotation.
4. Wash the beads 3 times with wash buffer (50mM Tris pH7.5, 150mM NaCl, 1% IGEPAL) or lysis buffer.
5. Resuspend the beads in 30 µl of **2x non-reducing** SDS sample buffer (125mM Tris pH6.8, 20% glycerol, 4% SDS, 0.005% Bromophenol blue).
6. Mix the beads by gently tapping the end of the tube, we do not recommend using a pipette for this. Incubate the solution at room temperature for 5 min. Collect supernatant by centrifuge tube at maximum speed for 1 min at 4°C.
7. Add 1 ul of beta mercaptoethanol to a new Eppendorf tube. Carefully remove sample from step 6 without disturbing the beads and transfer to the new tube containing beta mercaptoethanol. Boil sample for 5 min prior to loading on SDS-PAGE.
8. Run protein sample in SDS-PAGE.
9. Equilibrate the gel in Western transfer buffer (25mM Tris pH8.3, 192mM glycine, and 15% methanol) for 15 min at room temperature prior to electro-blotting.
10. Transfer protein to a PVDF membrane overnight (10-18h) at a constant 20 V.
11. Wash the membrane once with TBST.
12. Block the membrane with 3% non-fat dry milk in TBST for 1 h at room temperature with constant agitation.
13. Incubate the membrane with a primary antibody of your choice (e.g. For phosphotyrosine use APY03, 1:500 dilutioin in TBST) for 1-2 h at room temperature with constant agitation.
14. Wash the membrane 3 times with TBST for 10 min each.
15. Incubate the membrane with the appropriate secondary antibody. For example when using APY03 we use an anti-mouse secondary antibody (e.g. goat anti mouse HRP from Jackson Labs., Cat# 115-035-068) at 1:20000 in 3% non-fat dry milk for 60 min at room temperature with constant agitation.
16. Wash the membrane 6 times in TBST for 10 min each.
17. Use an enhanced chemiluminescence detection method to detect the signal (e.g. SuperSignal West Dura Extented Duration Substrate, Thermo Fisher).

References

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

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