Applications	Reactivity	Sensitivity	MW (kDa)	Source
W	M R	Endogenous	32	Rabbit

Applications Key: W=Western Blotting

Reactivity Key: M=Mouse R=Rat

Species cross-reactivity is determined by western blot. Species enclosed in parentheses are predicted to react based on 100% sequence homology.

Protocols

2301:

Western Blotting

Western Immunoblotting Protocol (Primary Ab Incubation In BSA)

For Western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4 ℃ with gentle shaking, overnight.

Products available from Cell Signaling Technology are linked by their respective catalog numbers.

A. Solutions and Reagents

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

1. **1X Phosphate Buffered Saline (PBS).**
2. **1X SDS Sample Buffer:** (#7722, #7723) 62.5 mM Tris-HCl (pH 6.8 at 25 ℃), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red.
3. **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5).
4. **10X Tris Buffered Saline (TBS):** (#9997) 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).
5. **Nonfat Dry Milk:** (#9999) (weight to volume [w/v]).
6. **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%).
7. **Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T).
8. **Bovine Serum Albumin (BSA):** (#9998).
9. **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%).
10. **Phototope®-HRP Western Blot Detection System:** (#7071 anti-rabbit) or (#7072 anti-mouse) Includes biotinylated protein ladder, secondary (#7074 anti-rabbit) or (#7076 anti-mouse) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO® chemiluminescent reagent and peroxide.
11. **Prestained Protein Marker, Broad Range (Premixed Format):** (#7720).
12. **Biotinylated Protein Ladder Detection Pack:** (#7727).
13. **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.

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 cm diameter 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 for complete cell lysis and to shear DNA (to reduce sample viscosity).
5. Heat a 20 µl sample to 95–100 ℃ for 5 minutes; cool on ice.
6. Microcentrifuge for 5 minutes.
7. 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.
8. Electrotransfer to nitrocellulose or PVDF membrane.

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) 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 three 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 ℃.
5. Wash three times for 5 minutes each with 15 ml of TBS/T.

I. For Unconjugated Primary Antibodies

1. Incubate membrane with **appropriate** 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.
2. Wash three times for 5 minutes each with 15 ml of TBS/T.

II. For HRP Conjugated Primary Antibodies

Skip to Detection of Proteins (Step D).

III. For Biotinylated Primary Antibodies

1. Incubate membrane with HRP-Streptavidin (at the appropriate dilution) in milk for one hour with gentle agitation at room temperature.
2. Wash three times for 5 minutes each with 15 ml of TBS/T.

D. Detection of Proteins

1. 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.
2. 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.

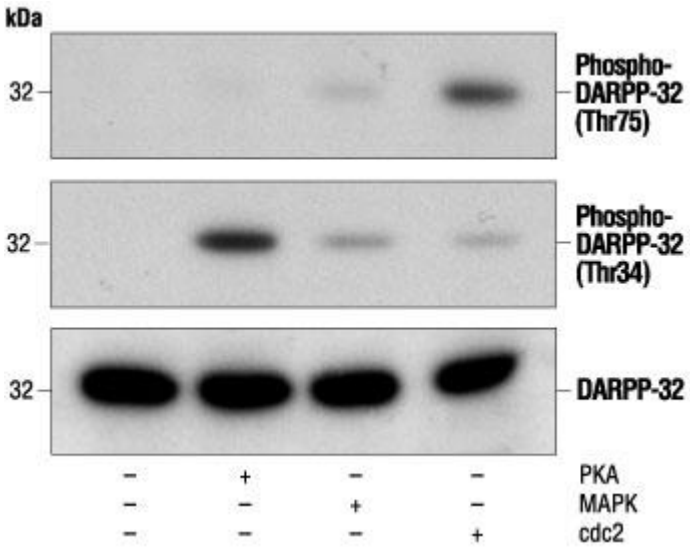
Specificity / Sensitivity

Phospho-DARPP-32 (Thr75) Antibody detects endogenous levels of DARPP-32 only when phosphorylated at threonine 75. The antibody does not cross-react with DARPP-32 phosphorylated at Thr34.

Source / Purification

Polyclonal antibodies are produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding Thr75 of human DARPP-32. Antibodies are purified by protein A and peptide affinity chromatography.

Western Blotting



Western blot analysis of extracts from rat brain cortex, untreated or phosphorylated in vitro by PKA, MAP kinase or cdc2 kinase, using Phospho-DARPP-32 (Thr75) Antibody #2301 (top), Phospho-DARPP-32 (Thr34) Antibody (middle) or DARPP-32 Antibody #2302 (bottom).

Background

DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein, relative molecular mass 32,000) is a cytosolic protein highly enriched in medium-sized spiny neurons of the neostriatum (1). It is a bifunctional signaling molecule that controls serine/threonine kinase and serine/threonine phosphatase activity (2). Dopamine stimulates phosphorylation of DARPP-32 through D1 receptors and activation of PKA. PKA phosphorylation of DARPP-32 at Thr34 converts it into an inhibitor of protein phosphatase 1 (1). DARPP-32 is converted into an inhibitor of PKA when phosphorylated at Thr75 by cyclin-dependent kinase 5 (CDK5) (2). Mice containing a targeted deletion of the DARPP-32 gene exhibit an altered biochemical, electrophysiological, and behavioral phenotype (3).

1. Nishi, A. et al. (1997) *J. Neurosci.* 17, 8147-8155.
2. Bibb, J.A. et al. (1999) *Nature* 402, 669-671.
3. Fienberg, A.A. et al. (1998) *Science* 281, 838-842.

Application References

- Danielli, B. et al. (2010) *J Neurochem* 112, 531-41. Applications: **Western Blotting**
- Scheggi, S. et al. (2007) *J Neurochem* 103, 1168-83. Applications: **Western Blotting**