NEUROMICS

Ubiquitin Datasheet

Catalog Number:	MO18001	**Host:**	Mouse
Product Type:	Mouse monoclonal antibody Clone: PD41	**Species Reactivity:**	Rat, Mouse, Human, Primate
Immunogen Sequence:	Full length ubiquitin of bovine origin	**Format:**	Liquid in 10mM sodium HEPES (pH7.5), 150mM NaCl, 100ug BSA and 50% glycerol

Applications: **Western blotting** 1:1000
Immunohistochemistry 1:750 (paraffin) 1:100 (free-floating)
Dilutions listed only as a recommendation. Optimal dilution should be determined by investigator.

Storage: Store at -20ºC. Do not aliquot.

Application Notes

Western Blot Protocol

Sample Preparation

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 plate). Immediately scrape cells from 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 (or PVDF) membrane.

Membrane Blocking and Antibody Incubations
Note: Volumes for 10 cm x 10 cm (100 cm^2) membrane; for different sized membranes, adjust vol. 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°C.
5. Wash three times for 5 minutes each with 15 ml of TBS/T.
6. Incubate membrane with HRP-conjugated secondary antibody (1:2000) in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature.
7. Wash three times for 5 minutes each with 15 ml of TBS/T.
8. Process membranes using enhanced chemiluminescence.

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Solutions and Reagents for Western Blot and Immunoprecipitaion
Note: Prepare solutions with Milli-Q or equivalently purified water.

1X Phosphate Buffered Saline (PBS)

1X SDS Sample Buffer:
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

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

1X Cell Lysis Buffer:
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 b-Glycerolphosphate, 1 mM Na3VO4, 1 µg/ml Leupeptin. *Note: Addition of PMSF before use is suggested.*

Transfer Buffer:
25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)

10X Tris Buffered Saline (TBS):
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).

Nonfat Dry Milk (weight to volume [w/v])

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%).

Wash Buffer:
1X TBS, 0.1% Tween-20 (TBS/T)

Bovine Serum Albumin (BSA)

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%).

Immunohistochemistry - Sodium Citrate antigen unmasking w/ TBS-T Wash Buffer

Deparaffinization/Rehydration

1. Deparaffinize/hydrate sections:
    a. Incubate sections in three washes of xylene for 5 minutes each.
    b. Incubate sections in two washes of 100% ethanol for 10 minutes each.
    c. Incubate sections in two washes of 95% ethanol for 10 minutes each.
2. Wash sections twice in dH2O for 5 minutes each.

Antigen Unmasking

3. For antigen unmasking, bring slides to boiling in 10 mM sodium citrate buffer pH 6.0 then maintain at a sub-boiling temperature for 10 minutes. Cool slides on bench top for 30 minutes.
4. Wash sections in dH2O three times for 5 minutes each.

Staining

5. Incubate sections in 3% hydrogen peroxide for 10 minutes.
6. Wash sections in dH2O three times for 5 minutes each.
7. Wash section in wash buffer for 5 minutes.
8. Block each section with 100–400 µl in dilution buffer for 1 hour at room temperature.
9. Remove solution and add 100–400 µl diluted primary antibody to each section. (Dilute antibody in dilution buffer.) Incubate overnight at 4°C.
10. Remove antibody solution and wash sections in wash buffer three times for 5 minutes each.
11. Add 100–400 µl secondary antibody to each section, diluted in dilution buffer according to manufacturer's recommendation. Incubate 30 minutes at room temperature.
12. If using ABC biotin/avidin method, make ABC reagent according to the manufacturer's instructions and incubate solution for 30 minutes at room temperature.
13. Remove secondary antibody solution and wash sections three times with wash buffer for 5 minutes each.
14. Add 100–400 µl ABC reagent to each section and incubate for 30 minutes at room temperature.
15. Remove ABC reagent and wash sections three times in wash buffer for 5 minutes each.
16. Add 100–400 µl DAB reagent to each section and monitor staining closely.
17. As soon as the section turns brown, immerse slides in dH2O.

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18. If desired, counterstain sections in hematotoxylin for 10 seconds.
19. Wash sections in dH2O three times for 5 minutes each.
20. Dehydrate sections:
    a. Incubate sections in 95% ethanol two times for 10 seconds each.
    b. Repeat in 100% ethanol, incubating sections two times for 10 seconds each.
    c. Repeat in xylene, incubating sections two times for 10 seconds each.
21. Mount coverslips.

Solutions and Reagents for Immunohistochemistry

Xylene, Ethanol, Distilled H2O (dH2O), Hematotoxylin

10X Tris Buffered Saline (TBS):
To prepare 1 L add 24.2 g Trizma base (C4H11NO3) and 80 g sodium chloride (NaCl) to 1 L dH2O. Adjust pH to 7.6 with concentrated HCl.

1X TBS/0.1% Tween-20 (wash buffer):
To prepare 1 L add 100 ml 10X TBS to 900 ml dH2O. Add 1 ml Tween-20 and mix

10 mM Sodium Citrate Buffer:
To prepare 1 liter, add 2.94 g sodium citrate to 1 liter dH2O. Adjust pH to 6.0.

3% Hydrogen Peroxide:
To prepare, add 30 ml 30% H2O2 to 270 ml dH2O.

Dilution Buffer:
5% normal horse, donkey or goat serum in wash buffer. Normal serum should be from same species as secondary antibody.

ABC Reagent (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA):
Prepare according to manufacturer's instructions 30 minutes before use.

DAB Reagent:
Add 6.7 µg of 30% hydrogen peroxide to 10 ml dH2O; add this mixture to 10 ml of 1 mg/ml DAB (diaminobenzidine tetrahydrochloride) in PBS, filter.

Immunohistochemistry Protocol for Floating Sections

Fix tissues by intracardiac perfusion with ice-cold PBS for 1 minute followed by ice-cold 4% paraformaldehyde in phosphate buffer delivered with a peristaltic pump at 50 ml/min for 10 minutes. Remove tissues and keep in the same fixative solution at 4°C for 24 hours, then section with a Vibratome at a thickness of 50 µm. Vibratome sections can be stored in an antifreeze solution at –20°C for at least several months.

ABC-DAB
1. Wash tissue sections in TBS/Triton.
2. Treat sections with freshly made 1% H2O2 (0.1 ml of 30% H2O2 in 3 ml TBS) for 30 minutes.
3. Wash the sections with TBS/Triton three times for 30 minutes each at room temperature.
4. Block nonspecific binding sites with 3% BSA in TBS/Triton for 30 minutes to 1 hour.
5. Incubate the sections with primary antibody diluted in
6. 3% BSA in TBS/Triton overnight at 4°C.
7. Wash the sections in TBS/Triton three times for 10 minutes each at room temperature.
8. Incubate the sections in biotinylated anti-rabbit secondary antibody (for polyclonal primaries) or biotinylated anti-mouse secondary antibody (for monoclonal primaries) diluted in 1%
9. BSA in TBS/Triton for 1 hour at room temperature.
10. Prepare ABC Reagent solution and leave it at room temperature for at least 15 minutes.
11. Wash the sections in TBS/Triton three times for 10 minutes each at room temperature.
12. Incubate the sections in the ABC reagent for 1 hour at room temp.
13. Wash the sections in TBS/Triton three times for 10 minutes each at room temperature.
14. Incubate the sections in DAB reagent until staining is optimal as determined by light microscopic examination. *Note: Handle DAB reagent with gloves.*
15. Wash the sections in TBS three times for 5 minutes each.
16. Mount the sections on gelatin-coated slides and dry them at room temperature.
17. Dehydrate the sections sequentially in 50%, 70%, 95% and100% ethanol for 2 minutes each, 50%:50% ethanol/xylenes for 2 minutes and 100% xylenes for 5 minutes.

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18. Mount the coverslides using Permount.

Fluorescence

1. Wash tissue sections in TBS/Triton.
2. Block nonspecific binding sites with 3% BSA in TBS/Triton for 30 minutes to 1 hour.
3. Incubate the sections with a first primary antibody (e.g., phospho--specific rabbit antibody) diluted in 3% BSA in TBS/Triton overnight at 4°C.
4. Wash the sections in TBS/Triton three times for 10 minutes each at room temperature.
5. Incubate the sections with a second primary antibody from a different species than the first primary antibody (e.g., mouse) diluted as above for 2 hours at 4°C.
6. Wash the sections in TBS/Triton three times for 30 minutes each at room temperature.
7. Incubate the sections in a fluorescent secondary antibody mixture containing fluorescence-labeled secondary antibody against the first primary (i.e., fluorescence-labeled anti-rabbit) and fluorescence-labeled secondary antibody against the second primary antibody (i.e., fluorescence-labeled anti-mouse) each at a dilution of 1:200 in 1% BSA in TBS/Triton for 1 hour at room temperature. Incubation chambers should be covered with foil to avoid exposure to light.
8. Wash the sections in TBS three times for 30 minutes each at room temperature.
9. Mount and coverslip the sections using Gelvatol. Add a small drop of Gelvatol to sections. Carefully place coverslips on the drops, avoiding air bubbles.
10. The mounting media will set overnight at 4°C or within 2–3 hours at room temperature.

Solutions and Reagents

Phosphate Buffer:
0.1 M Na2HP04/NaH2P04 (pH 7.5)

Fixative Solution:
Phosphate buffered saline (PBS), 4% paraformaldehyde in phosphate buffer

Antifreeze Solution:
320 ml 1X PBS (pH 7.4), 240 ml ethylene glycol (30%), 240 ml glycerol (30%)

Tris Buffered Saline (TBS):
0.1 M Tris-HCl (pH 7.4), 0.15 M NaCl

Wash Buffer:
1X TBS, 0.1% Triton X-100 (TBS/Triton)

1% and 3% Bovine Serum Albumin (BSA)

ABC Reagent (avidin-biotin-peroxidase complex):
(Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) 1 drop reagent A and 1 drop reagent B in 5 ml TBS with 1% BSA, 0.1% Triton X-100

DAB Reagent (0.5 mg/ml):
Use 10 ml TBS, 500 µl of 10 mg/ml DAB stock solution, 50 µl of glucose oxidase (30 mg/10 ml TBS), 20 µl NH4Cl (2.0 g/10 ml TBS) and 50 µl D (+) glucose (2.5 g/10 ml TBS).

Gelvatol Preparation:
1) Add 2.4 g of polyvinyl alcohol (Mol. Wt. 30,000–70,000) to 6 ml of glycerol. Stir well to mix. Add 6 ml of dH2O and leave for at least 2 hours at room temperature.
2) Add 12 ml of 0.2 M Tris (pH 8.5). Heat to 50°C for 10 minutes with occasional mixing. After polyvinyl alcohol is dissolved, clarify by centrifugation (5000 x g) for 15 minutes. Collect supernatant liquid.
3) Add DABCO (1,4-diazabicyclo [2.2.2] octane; Sigma #D2522) to 2.5% as antifade medium. Aliquot in microtubes and store at –20°C. Stocks of Gelvatol are stable at room temperature for several weeks after thawing.

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