Thimet oligopeptidase (IVD6) Mouse mAb

100 µl
(10 Western mini-blots)

**Applications**
- W, IHC-P

**Species Cross-Reactivity**
- H, Mk

**Molecular Wt.**
- 80 kDa

**Source**
- Mouse

**Isotype**
- IgG2B

**Background:** Amyloid beta precursor protein (APP) is a 100-140 kDa transmembrane glycoprotein containing the amyloid domain (Aβ). β-secretase is involved in proteolytic cleavage of APP releasing Aβ. The extracellular deposition and accumulation of the released Aβ fragments form the main components of amyloid plaques in Alzheimer's disease. Thimet oligopeptidase is a ubiquitously expressed metalloendopeptidase that was originally identified as a candidate β-secretase (2). However, subsequent studies show that Thimet oligopeptidase promotes Aβ degradation. In addition, Thimet oligopeptidase inactivates a number of neuropeptides such as somatostatin, bradykinin, substance P and neurotensin. Finally, Thimet oligopeptidase has been shown both to increase and decrease presentation of antigenic peptides by the MHC-I at the cell surface. Despite these opposing results, there is consensus that Thimet oligopeptidase degrades peptides released by the proteasome (4–5).

**Specificity/Sensitivity:** Thimet oligopeptidase (IVD6) Mouse mAb detects endogenous levels of total Thimet oligopeptidase protein.

**Source/Purification:** Monoclonal antibody is produced by immunizing mice with a synthetic peptide (KLH-coupled) corresponding to residues in the amino-terminal region of Thimet oligopeptidase protein.

**Selected Application References:**

**Recommended Antibody Dilutions:**
- Western blotting: 1:1000
- Immunohistochemistry (Paraffin): 1:25
- Immunoprecipitation: 1:1000

**Immunohistochemical analysis of paraffin-embedded human Alzheimer brain, using Thimet Oligopeptidase (IVD6) Mouse mab.**

**Western blot analysis of extracts from HeLa and SH-SY5Y cells, using Thimet oligopeptidase (IVD6) Mouse mAb.**

**Background References:**
Western Immunoblotting Protocol (Primary Ab Incubation In Milk)

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

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: 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
3. Transfer Buffer: 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
4. 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).
5. Nonfat Dry Milk (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)
9. Primary Antibody Dilution Buffer: 1X TBS, 0.1% 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 20 µl Tween-20 (100%).
10. Phototope®-HRP Western Blot Detection System #7072: Includes biotinylated protein ladder, secondary anti-mouse (#7076) 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 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).

NOTE: CST recommends loading prestained molecular weight marker (#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 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 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 with 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.
7. 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.
**A Solutions and Reagents**

1. Xylene
2. Ethanol, anhydrous denatured, histological grade (100% and 95%)
3. Deionized water (dH₂O)
4. Hematoxylin (optional)

5. **Wash Buffer:**
   - 1X TBS/0.1% Tween-20 (1X TBST): To prepare 1L add 100 ml 10X TBS to 900 ml dH₂O. Add 1 ml Tween-20 and mix.
   - 10X Tris Buffered Saline (TBS): To prepare 1 L add 24.2 g Trizma® base (C₆H₃N₃O₅) and 80 g sodium chloride (NaCl) to 1 L dH₂O. Adjust pH to 7.6 with concentrated HCl.

6. **Antigen Unmasking:**
   - 3% Hydrogen Peroxide: To prepare, add 10 ml 30% H₂O₂ to 90 ml dH₂O. Adjust pH to 6.0.
   - 10 mM Sodium Citrate Buffer: To prepare 1 L add 24.2 g sodium citrate trisodium salt dihydrate (C₆H₃N₃O₅.2H₂O) and 14.4 g sodium phosphate, dibasic (Na₂HPO₄) and 2.4 g potassium phosphate, monobasic (KH₂PO₄) to 1 L dH₂O. Adjust pH to 7.4.

7. **Antigen Unmasking:**
   - Citrate: 10 mM Sodium Citrate Buffer: To prepare 1 L add 2.94 g sodium citrate trihydrate (C₆H₃N₃O₅.2H₂O) to 1 L dH₂O. Adjust pH to 6.0.
   - EDTA: 1 mM EDTA: To prepare 1 L add 0.372 g EDTA (C₆H₈N₂O₅) to 1 L dH₂O. Adjust pH to 8.0.
   - TE: 10 mM Tris/1 mM EDTA/0.059 Tween–20, pH 9.0: To prepare 1L add 1.21 g Trizma® base (C₆H₃N₃O₅) and 0.372 g EDTA (C₆H₈N₂O₅) to 950 ml dH₂O. Adjust pH to 9.0, add 0.5 ml Tween–20, then adjust final volume to 1000 ml with dH₂O.

8. **Blocking Solution:**
   - Normal Goat Serum (5%): To prepare, add 10 ml 10% normal goat serum to 90 ml dH₂O.

**B Deparaffinization/Rehydration**

**NOTE:** Do not allow slides to dry at any time during this procedure.

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.

**C *Antigen Unmasking***

**NOTE:** Consult product data sheet for specific recommendation for the unmasking solution.

1. For Citrate: Bring slides to a boil 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.
2. For EDTA: Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 minutes at a sub-boiling temperature. No cooling is necessary.
3. For TE: Bring slides to a boil in 10 mM TE/1mM EDTA/0.059 Tween-20, pH 9.0 then maintain at a sub-boiling temperature for 18 minutes. Cool on the bench for 30 minutes.
4. For Pepsin: Digest for 10 minutes at 37°C.

**D Staining**

1. Wash sections in dH₂O three times for 5 minutes each.
2. Incubate sections in 3% hydrogen peroxide for 10 minutes.
3. Wash sections in dH₂O twice for 5 minutes each.

**NOTE:** Consult product data sheet for recommended antibody diluent.

4. Wash section in wash buffer for 5 minutes.
5. Block each section with 100-400 µl blocking solution for 1 hour at room temperature.
6. Remove blocking solution and add 100-400 µl primary antibody diluted in recommended antibody diluent to each section. Incubate overnight at 4°C.
7. Remove antibody solution and wash sections in wash buffer three times for 5 minutes each.
8. Add 100-400 µl biotinylated secondary antibody, diluted in TBST per manufacturer’s recommendation, to each section. Incubate 30 minutes at room temperature.
9. If using ABC avidin/biotin method, prepare ABC reagent according to the manufacturer's instructions and incubate solution for 30 minutes at room temperature.
10. Remove secondary antibody solution and wash sections three times with wash buffer for 5 minutes each.
11. Add 100-400 µl ABC reagent to each section and incubate for 30 minutes at room temperature.
12. Remove ABC reagent and wash sections three times in wash buffer for 5 minutes each.
13. Add 100-400 µl DAB or suitable substrate to each section and monitor staining closely.
14. As soon as the sections develop, immerse slides in dH₂O.
15. If desired, counterstain sections in hematoxylin per manufacturer’s instructions.
16. Wash sections in dH₂O two times for 5 minutes each.
17. 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.
18. Mount coverslips.