



**Anti-Conjugated NO-L-Cysteine
MOUSE MONOCLONAL
AB-T125**

Example of ELISA protocol used to test conjugated NO-L-Cystein:

1. Coating of conjugated NO-L-Cystein ($15\mu\text{g/ml}$) in maxisorp well plates (Nunc) with a solution of sodium carbonate buffer 0.05M (pH 9.6), during sixteen hours at 4°C .
2. Saturation of well plates with of a solution of PBS (pH 7.3) containing 2.5g/l of BSA (Acros) and 0.05% Tween 20 (Acros) during one hour at 37°C .
3. Wash with PBS Tween (two times).
4. Anti-conjugated NO-L-Cystein antibody will be diluted ($1/1,000$ - $1/5,000$) in PBS containing 2.5g/l BSA and 10% of glycerol, $200\mu\text{l}$ by well plate (incubating during 2 hours at 37°C).
5. Wash with PBS Tween (three times).
6. $200\mu\text{l}$ of peroxidase-labeled goat anti-mouse (Sigma) diluted ($1/10,000$) in a solution of PBS containing 2.5g/l BSA, 10% of glycerol and 0.5% of Tween, will be applied by well plate (during one hour at 37°C).
7. Well plates will be rinsed with a PBS Tween (three times).
8. And finally the peroxidase will be developed by incubating $200\mu\text{l}$ by well plate of a citrate 0.1M /phosphate 0.2M (pH 5) solution containing 0.4% of OPD (Sigma) and 0.03% of hydrogen peroxide (Acros) for ten minutes in the dark, after that, we will stop the reaction by the addition of $50\mu\text{l}$ of 2M HCl.
9. The optical density will be measured at 492nm , to obtain the different values (IC 50).

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Example of Immunohistochemistry protocol

Perfusion protocol for Adult male Sprague Dawley (weight around 0.5 kg) :

1. The animals can be deeply anesthetized for example with urethane (0.5-1.5g/kg, intraperitoneal).
2. Heparinized, and perfused via the ascending aorta with 100 ml of cold physiologic saline (0.9% NaCl) and with the following fixative solution:
 - a) 300 ml of cold 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate-buffer (PB), pH 7.2, (twominutes).
 - b) 600 ml of cold 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate-buffer (PB), pH 7.2, (tenminutes).
 - c) Dissect out the brain and place in a solution of 4% paraformaldehyde in 0.1M PB, pH 7.2, at 4°C for 12-16 hours.
 - d) Before the brains are cut on a freezing microtome, treat the brain in growing concentrations of sucrose (a first solution of 5% of sucrose in PBS until brain sinks), after that, repeat the same process in a solution with a higher level of sucrose (10%), 20%, 25% and finally 30%.

Around 50 μ m-thick serial sections should be obtained, kept at 4°C in PBS (0.1 M, pH 7.2) and processed for immunostaining.

Example of immunohistochemical protocol :

1. In order to avoid possible interference with endogenous peroxidase, free-floating sections are treated with distilled water containing NH₃ (20%), H₂O₂ (30%) and NaOH (1%) for 20 min (another method is using a solution with 33% of H₂O₂ and 66% of methanol).
2. Wash the sections for 20 min in 0.15 M phosphate-buffered saline (PBS) (pH 7.2)
3. Pre-incubate for 30 min in PBS containing 10% normal horse serum and 0.3% of Triton X-100 (mixed solution).
4. Incubate at room temperature (1h 30min) and overnight at 4°C in the same mixed solution containing anti-conjugated NO- L-Cysteine antibody (diluted 1/1,000 to 1/5,000; as recommended dilution).
5. Wash the sections in PBS (30 min).
6. Incubate for 60 min at room temperature with biotinylated anti-mouse immunoglobulin (Vector) diluted 1/200 in PBS.
7. Wash the sections in PBS (30 min).
8. Incubate sections for 1 h with a 1/100 diluted avidin-biotin-peroxidase complex (Vectastain).
9. Wash the sections in PBS (30 min).
10. Wash with Tris-HCl buffer, pH 7.6 (10 min).
11. Develop tissue-bound peroxidase with H₂O₂ using 3, 3' diaminobenzidine as chromogen.
12. Rinse sections with PBS and coverslip with PBS/Glycerol (1/1).



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Example of Western blot protocol

Membrane Blocking, Antibody Incubations and Detection

1. Saturate the blot membrane with TBS + 5% Blocker for 1 hour at 37°C while mixing
2. Wash the membrane twice for 5 minutes in TBS Tween at 37°C
3. Incubate the membrane with anti-NO-L-Cysteine antibody diluted 1:1000 in TBS 0.5% Blocker for 2 hours at 37°C
4. Wash the membrane three times for 5 minutes in TBS Tween at 37°C
5. Incubate with a biotinylated secondary antibody diluted (1/1,000-1/2,000) in TBS 0.5% Blocker for 2 hours at 37°C
6. Wash the membrane three times for 5 minutes in TBS Tween at 37°C
7. Incubate with Streptavidin-HRP 1µg/ml in TBS 0.5% Blocker for 2 hours at room temperature
8. Wash the membrane three times for 5 minutes in TBS at 37°C
9. Incubate in TBS (200ml) + (50mg DAB in 25ml methanol) + (150mg 4-chloro-1-naphtol in 25ml methanol) + 50µl H₂O₂ 30% for a maximum of 30 minutes in the dark
10. Stop the reaction by addition of distilled water

Blocker = skim milk (Biorad 170-6404)

TBS = 20mM Tris base, 0.5M NaCl, pH 7.5

TBS Tween = TBS + 0.05% Tween 20