



11175-A FLINTKOTE AVENUE, SAN DIEGO, CA 92121
TELEPHONE (858) 642-1988 • FAX (858) 642-1989
WWW.ATSBIO.COM • ATS@ATSBIO.COM

**Anti-Conjugated Pyridoxine
RAT POLYCLONAL**

Catalog Number: AB-T144

ELISA protocol used to test conjugated Pyridoxine:

1. Coating of conjugated pyridoxine ($15\mu\text{g/ml}$) 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 phosphate buffer saline (PBS) (pH 7.3) containing 2g/l of BSA (Acros) during one hour at 37°C .
3. Wash with PBS (three times).
4. Preabsorbed pyridoxine antiserum will be diluted ($1/2,000$ - $1/5,000$) in PBS containing 2g/l BSA and 5% of glycerol, $200\mu\text{l}$ by well plate (incubating during 2 hours at 37°C).
5. Wash with PBS (three times).
6. $200\mu\text{l}$ of peroxidase-labeled goat anti-rat (Jackson) diluted ($1/5,000$) in a solution of PBS containing 5g/l of BSA, will be applied by well plate (during one hour at 37°C).
7. Well plates will be rinsed with a PBS solution containing 0.5% of Tween.
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 .

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Example of Immunohistochemistry:

Perfusion protocol for Adult male monkeys (Macaca fascicularis) (weight 3-3.5 kg):

1. The animals can be deeply anaesthetized with ketamine (8mg/kg, intramuscular) and sodium thiopental (500 mg/kg, intraperitoneal).
2. Heparinized, and perfused via the ascending aorta with 300ml of cold physiologic saline (0.9% NaCl) and with the following fixative solutions:
 - a) 500ml of 1% paraformaldehyde in 0.1M phosphate-buffer (PB), pH 7.2, at room temperature (two minutes).
 - b) 2,500ml of 4% paraformaldehyde in 0.1M PB, pH 7.2, at 4°C (ten minutes).
 - c) 5,000ml of cold 4% paraformaldehyde in 0.1M PB, pH 7.2 (fifty minutes).
 - d) 2,000ml of cold 5% sucrose in 0.1M PB, pH 7.2 (twenty minutes).
 - e) Dissect out the brains and place in 10% glycerol and 2% dimethylsulfoxide (DMSO) in 0.1M PB, pH 7.2, at 4°C for two days, and finally keep at the same temperature in 20% of glycerol and 2% DMSO in PB until the brains will be cut on a freezing microtome.

Around 50µm-thick serial sections will be obtained, kept at 4°C in PB (0.1 M, pH 7.2) containing 20% of glycerol and 30% of ethylene glycol, and processed for immunostaining.

Example of Immunohistochemical Protocol:

1. In order to avoid possible interference with endogenous peroxidase, free-floating sections will be treated with distilled water containing NH₃ (20%), H₂O₂ (30%) and NaOH (1%) for 20 min (other method is using a solution with 33% of H₂O₂ and 66% of methanol).
2. Then, 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% of 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 Pyridoxine antiserum (diluted 1/1000–1/2,000; as recommended dilutions).
5. Then, the sections will be wash in PBS (30 min).
6. After that we will incubate for 60 min at room temperature with biotinylated anti-rat immunoglobulin (Vector) diluted 1/200 in PBS.
7. Wash during 30 min with PBS.
8. Sections will be incubated for 1 h with a 1/100 diluted avidin-biotin-peroxidase complex (Vectastain) in the mixed solution.
9. After that we will wash the sections in PBS (30 min).
10. Wash with Tris-HCl buffer (pH 7.6)(10 min).
11. The tissue-bound peroxidase will be developed with H₂O₂ using 3, 3' diaminobenzidine as chromogen.
12. Finally the sections will be rinsed with PBS and coverslipped with PBS/Glycerol (1/1).