



cell signaling solutions

Certificate of Analysis

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Anti-Nitrotyrosine

(rabbit immunaffinity purified IgG)

Catalog # 06-284

Lot # 23446

Immunogen: Nitrated KLH.

Formulation: 100µg of immunaffinity purified rabbit IgG in 182µl of 0.2M Tris-glycine, pH 7.4, 0.15M NaCl, 0.1mM EDTA, 0.05% sodium azide and 10mg/ml BSA. Frozen solution.

Storage and Stability: Stable for 1 year at -20°C from date of shipment. Aliquot to avoid repeated freezing and thawing. For maximum recovery of product, centrifuge the original vial after thawing and prior to removing the cap.

**FOR RESEARCH USE ONLY
NOT FOR USE IN HUMANS**

Quality Control Testing

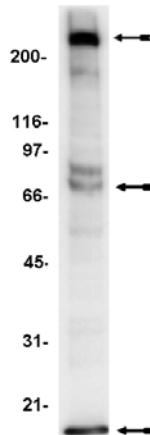
Immunoblot Analysis: 0.5-2µg/ml of this lot detected nitrotyrosine immunoblotting controls (Catalog # 12-354) at 16kDa, 66kDa and 215kDa. Occasionally an approximate 32kDa band will be seen representing a dimer of the 16kDa protein.

Immunocytochemistry: 5-10µg/ml of this lot detected nitrated proteins from human A431 cells pretreated 5 minutes with 24mM peroxyxynitrite (Catalog # 20-107).

Immunohistochemistry: 5µg/ml of a previous lot detected nitrated proteins in formalin-fixed, paraffin embedded lateral ischemic rat brain tissue.

Additional Research Applications

Immunohistochemistry: This antibody has been reported to detect nitrotyrosine containing proteins in acutely injured human lung tissue.¹⁻⁵



Immunoblot Analysis

Representative blot from a previous lot. Nitrotyrosine immunoblotting controls were resolved by electrophoresis, transferred to nitrocellulose and probed with anti-Nitrotyrosine (2µg/ml). Proteins were visualized using a goat anti-rabbit secondary antibody conjugated to HRP and a chemiluminescence detection system. Arrow indicates nitrotyrosine molecular weight standards.

Application References:

1. Beckman, J.S., *et al.*, Biol. Chem. Hoppe-Seyler **375**: 81-88 1994.
2. Ohshima, H., *et al.*, Fund. Chem. Tox. **28**: 647-652, 1990.
3. Ischiropoulos, H., *et al.*, Arch. Biochem. Biophys. **298**: 431-437, 1992.
4. Beckman, J.S., *et al.*, Nature **364**: 584, 1993.
5. Kooy, N.W., *et al.*, Am. J. Respir. Crit. Care Med. **151**: 1250-1254, 1995.

Immunoblot Protocol

1. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 1:1 dilution of Laemmli reducing sample buffer and nitrosylated tyrosine molecular immunoblot controls, or a peroxyxynitrite treated cell lysate, and transfer the proteins to nitrocellulose. Wash the blotted nitrocellulose twice with water.
2. Wash the nitrocellulose in PBS-0.05% Tween-20 (PBS-T) for 10 minutes.
3. Wash the nitrocellulose with water twice.
4. Block the blotted nitrocellulose in freshly prepared 3% nonfat dry milk (Catalog # 20-200) in PBS (PBS-MLK) for 20-30 minutes at room temperature with constant agitation.
5. Wash the nitrocellulose with water twice.
6. Incubate the nitrocellulose with **0.5-2µg/ml of anti-Nitrotyrosine** diluted in freshly prepared PBS-MLK overnight with agitation at 4°C.
7. Wash the nitrocellulose with water five times.
8. Incubate the nitrocellulose in the secondary reagent of choice (a goat anti-rabbit HRP conjugated IgG, Catalog # 12-348, 1:5000 dilution was used) in PBS-MLK for 1.5 hours at room temperature with agitation.
9. Wash the nitrocellulose with water five times.
10. Wash the nitrocellulose in PBS-T for 5 minutes.
11. Wash the nitrocellulose with water five times.
12. Use detection method of choice: enhanced chemiluminescence is recommended.

Immunocytochemistry Protocol

1. Plate approximately 200µl of cell suspension.
2. Incubate 24 hours in a 37°C CO₂ incubator.
3. Wash the cells three times for 5 minutes with PBS. Do not shake cells.
4. Fix the cells with ethanol:acetic acid [95:5] for 1 minute.
5. Wash the cells with PBS, twice, for 5 minutes. Do not shake.
6. Add 200µl of 24mM peroxyxynitrite to the positive control wells for 5 minutes.
7. Wash the cells with PBS, twice, for 15 minutes. Do not shake.
8. Cover cells with 400µl of 1% BSA in PBS and incubate for 30 minutes at room temperature.
9. Wash the cells with PBS, twice, for 15 minutes. Do not shake.
10. Incubate the cells with **5-10µg/ml anti-Nitrotyrosine** in 1% BSA in PBS for 2 hours at room temperature or overnight at 4°C.
11. Wash the cells with PBS, twice, for 5 minutes. Do not shake.
12. Incubate the cells with a 1:200 dilution of goat anti-rabbit IgG fluorescein conjugated secondary antibody in PBS for 1.5 hours at room temperature, in the dark.
13. Wash the cells three times with PBS, three times, for 5 minutes in the dark. Do not shake.
14. Mount and examine the cells under a fluorescent microscope.

Immunohistochemistry Protocol

- A. Deparaffinize section and rehydrate as follows: Three times for 5 minutes in xylene; two times for 5 minutes in 100% ethanol; two times for 5 minutes in 95% ethanol; and once for 5 minutes in 80% ethanol.
- B. High temperature antigen retrieval
 1. Place sections in a Coplin jar with dilute antigen retrieval solution of choice (10mM citrate, pH 6).
 2. Place Coplin jar containing slides in vessel filled with water and microwave on high for 2 - 3 minutes (700 watt oven).
 3. Check level of retrieval solution, allow to cool for 2 - 3 minutes and repeat steps 2 and 3, four times (depending on tissue).
 4. Remove Coplin jar containing sections and allow to cool for 20 minutes at room temperature.
 5. Rinse sections in deionized water, two times for 5 minutes.
 6. Place slides in endogenous oxidation blocking solution (PBS + 2% hydrogen peroxide).
 7. Rinse slides once for 5 minutes in PBS.

C. Blocking and staining

1. Block all sections with PBS/1% bovine serum albumin (PBA) for 1 hour at room temperature.
2. Incubate sections in 2% normal serum diluted in PBA for 30 minutes at room temperature to reduce immunologic binding of antibody. Perform the incubation in a sealed, humidity chamber to prevent air-drying of the tissue sections. (The choice of blocking serum is the same as the species of the biotinylated secondary antibody).
3. Gently shake off excess PBA and cover sections with **5µg/ml anti-Nitrotyrosine** diluted in PBA and incubate either at room temperature for 1 hour or overnight at 4°C. (Care should be taken that the sections do not touch during incubation).
4. Rinse sections twice for 5 minutes in PBS, shaking gently.
5. Gently remove excess PBS and cover sections with diluted biotinylated secondary antibody in PBA for 30 minutes - 1 hour at room temperature in the humidity chamber.
6. Rinse sections twice for 5 minutes in PBS, shaking gently.
7. Remove excess PBS and incubate for 1 hour at room temperature in an avidin-biotin detection system.
8. Rinse twice for 5 minutes in PBS, shaking gently.

D. Develop and counterstain

1. Incubate sections for approximately 2 minutes in peroxidase substrate solution made up immediately prior to use as follows:
 - 10mg diaminobenzidine (DAB) dissolved in 10ml 50mM sodium phosphate buffer, pH 7.4.
 - 12.5µl 3% CoCl₂/NiCl₂ in deionized water.
 - 1.25µl hydrogen peroxide.

Note: Special precautions should be taken when handling DAB because of its possible carcinogenic properties!

2. Rinse slides well three times for 10 minutes in deionized water.
3. Counterstain with 0.01% Light Green acidified with 0.01% acetic acid for 1-2 minutes depending on intensity of counterstain desired.
4. Rinse slides three times for 5 minutes with deionized water.
5. Dehydrate two times for 2 minutes in 95% ethanol; two times for 2 minutes in 100% ethanol and two times for 2 minutes in xylene.
6. Mount slides.

Note: It is recommended to run both a positive and negative control.

Negative control: Incubate the primary antibody with 10mM nitrotyrosine in PBS for 1 hour at room temperature. Use this solution in place of the primary antibody in the above protocol (step C3)

Positive control: After the slides have been deparaffinized, incubate the slides for 20 minutes at room temperature in the following solution: 1mM sodium nitrite, 1mM hydrogen peroxide in 100mM acetate buffer, pH 5.0. The slides are then processed using the above protocol.