# Nitrotyrosine IP Kit

Item No. 601220

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Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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# GENERAL INFORMATION

## Materials Supplied

| Item Number | Item | Quantity/Amount | Storage |
|---|---|---|---|
| 601225 | Nitrotyrosine Affinity Sorbent | 1 vial/400 µl | 4°C |
| 601221 | Nitrotyrosine Polyclonal Antibody | 1 vial/150 µl | -20°C |
| 601222 | Nitrotyrosine BSA | 1 vial/5 µg | -20°C |
| 601223 | Spin Columns and Collection Tubes | 10 each | RT |

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

**WARNING:** THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

## Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

## Precautions

**Please read these instructions carefully before beginning this assay.**

## If You Have Problems

**Technical Service Contact Information**

| | |
|---|---|
| **Phone:** | 888-526-5351 (USA and Canada only) or 734-975-3888 |
| **Fax:** | 734-971-3641 |
| **Email:** | techserv@caymanchem.com |
| **Hours:** | M-F 8:00 AM to 5:30 PM EST |

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

## Storage and Stability

This kit will perform as specified if stored as directed on page 3 and used before the expiration date indicated on the outside of the box. *NOTE: This kit ships at 4°C. Item Nos. 601221 and 601222 should be removed and stored at -20°C for best results.*

## Materials Needed But Not Supplied

1. Microfuge tubes (1.5 ml)
2. Cell Lysis Buffer with protease inhibitor
3. Wash Buffer: Cell Lysis Buffer without protease inhibitor
4. Elution Buffer (as appropriate)
   - SDS-PAGE Sample Loading Buffer - Western blot (WB) analysis
   - 0.1% Formic acid - Proteomic analysis
5. Target protein specific primary antibodies
6. Anti-rabbit AP or HRP conjugated secondary antibody (if using the Nitrotyrosine Polyclonal Antibody provided in the kit).
7. Reagents for WB

# INTRODUCTION

## Background

Nitric oxide (NO) is a product of the enzymatic conversion of arginine to citrulline by nitric oxide synthase (NOS). NO reacts rapidly with superoxide (6.7 x $10^9$ $M^{-1}sec^{-1}$) to form peroxynitrite. At physiological pH and in the presence of transition metals, peroxynitrite undergoes heterolytic cleavage to form hydroxyl anion and nitronium ion, the latter of which nitrates protein tyrosine residues. Thus, the presence of nitrotyrosine on proteins can be used as a marker for peroxynitrite formation *in vivo*.[1,2] Nitrotyrosine has been shown to be present in proteins from a variety of clinical conditions including atherosclerotic lesions of human coronary arteries, postischemic heart, and placenta during preeclampsia.[3,4,5] Increased nitration of proteins in motor neurons has been identified in patients with amyotrophic lateral sclerosis (ALS) and may be due to mutations in superoxide dismutase.[2,5-8]

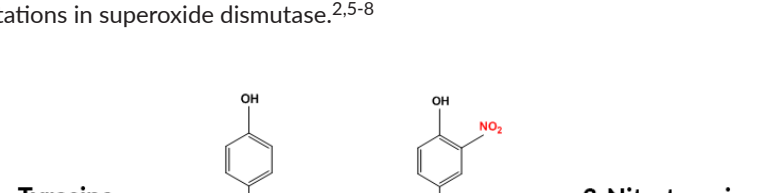



**Figure 1. Structure of 3-Nitrotyrosine**

## About This Assay

Cayman's Nitrotyrosine IP Kit allows for the capture and pulldown of nitrated proteins from cell lysates using a sorbent coupled with a nitrotyrosine monoclonal antibody. The nitrated proteins can be eluted from the resin, loaded to an SDS-PAGE gel, and probed using the included Nitrotyrosine Polyclonal Antibody or a user supplied target specific antibody. A positive control sample assures that the assay is working.

1. Determine change in tyrosine nitration in treated *versus* non-treated samples.
2. Demonstrate target proteins are nitrated *in vitro*
3. Identify and characterize nitrated proteins by WB and proteomic analysis.

# PRE-ASSAY PREPARATION

## Reagent Preparation

*All reagents unless listed below are ready to use as supplied.*

**Lysis Buffer**

- Assay is compatible with a wide range of lysis buffers
- Avoid buffer components that cause protein denaturation
- Minimize use of reducing agents (*e.g.*, DTT) and detergents where possible
- Suggested Lysis Buffer: 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% (v/v) NP-40, and protease inhibitor cocktail.

**Elution Reagent**

Select appropriate eluting reagent for intended method of analysis:

a) SDS-PAGE Sample Loading Buffer - WB analysis

b) 0.1 % Formic acid - Proteomic analysis

## Sample Preparation

Recommend using 200 µl sample/control lysate at 1-5 mg/ml per assay as a starting point. Adjust lysate concentration with Lysis Buffer if required.



# ASSAY PROTOCOL

## Performing the Assay

**Assay Optimization**

Optimal assay conditions for capture of nitrotyrosine containing proteins from specific lysate samples must be determined by the user. Adjustment of the following parameters may facilitate this process:

- Sample volume, 100-500 µl
- Sample concentration, 1-5 mg/ml
- Nitrotyrosine Affinity Sorbent volume, 40 µl suspension (20 µl settled resin)
- Assay time, minimum 4 hours to overnight

***Keep reaction components on ice throughout set-up.***

### Nitrotyrosine Affinity Sorbent Preparation

1. Re-suspend the Nitrotyrosine Affinity Sorbent by gentle inversion of the tube
2. Aliquot 40 µl Nitrotyrosine Affinity Sorbent suspension (20 µl settled resin) into required number of Spin Columns
3. Add 500 µl Wash Buffer to each column
   a. Mix with pipette
   b. Centrifuge at low speed (500-1,000 x g, for one minute) to collect matrix
   c. Discard flow through
4. Repeat matrix wash/collection at least twice

## Nitrotyrosine Affinity Sorbent Assay

5. Add 200 µl sample/control lysate to Nitrotyrosine Affinity Sorbent and mix with pipette
6. Incubate for a minimum two hours at 4°C with rotary mixing
7. Centrifuge at low speed (500-1,000 x g, for one minute) to collect matrix
8. Remove flow through and retain as 'Unbound Fraction' for subsequent analysis if required
9. Replace column in collection tube
10. Wash matrix by adding 500 µl Wash Buffer to column
    - Centrifuge at low speed (500-1,000 x g, for one minute) to collect matrix
        i. Repeat wash step two more times

## Elution of captured proteins

11. For SDS-PAGE/WB analysis:
    - Add 20 µl of SDS-PAGE Sample Buffer to Spin Column and mix with pipette
    - Place column in microfuge tube
    - Heat to 95°C for five minutes
    - Centrifuge at low speed (500-1,000 x g, for one minute) to collect eluted materials
    - Analyze or store at -20°C
12. For Proteomic analysis:
    - Add 10 volumes (200 µl) 0.1% formic acid to Spin Column
    - Rotary mix for 5-10 minutes at room temperature
    - Place column in microfuge tube
    - Centrifuge at low speed (500-1,000 x g, for one minute) to collect eluted materials
    - Eluate can then be lyophilized and re-suspended in trypsin digestion or alternative buffer prior to subsequent processing/analysis, or stored at -20°C

# ANALYSIS

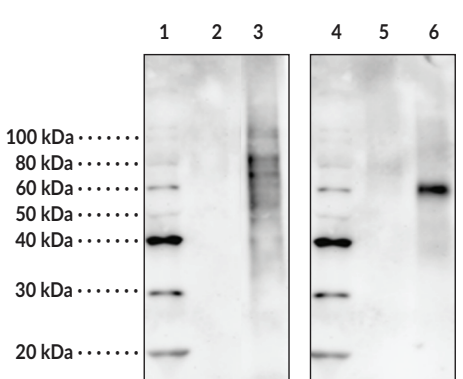
## Performance Characteristics

### Western Blot Analysis

The Nitrotyrosine IP Kit contains a rabbit polyclonal anti-nitrotyrosine antibody for analysis by WB.

| Variable | Recommendation |
|---|---|
| SDS-PAGE | 10-20% gel |
| Sample loading | 10 µl quenched reaction |
| Anti-Nitrotyrosine pAb | 1:200 dilution |
| Nitrotyrosine BSA | 100 ng |
| Secondary Antibody (user supplied) | Goat Anti-Rabbit (HRP Conjugate) |
| Target protein specific antibody (user supplied) | WB conditions must be determined by the user and the Antibody applied in conjunction with an appropriate Secondary Antibody |

### Example Assay Results




**Lane 1:** MW Markers
**Lane 2:** Unmodified Raji Cell Lysates
**Lane 3:** Nitrated Raji Cell Lysates
**Lane 4:** MW Markers
**Lane 5:** Unmodified BSA
**Lane 6:** Nitrated BSA

**Figure 2. Western blot analysis of Nitrotyrosine IP Kit.** Peroxynitrite-treated and control cell lysates as well as the supplied positive control were run on a 12% gel, transferred to nitrocellulose, and probed with the provided Anti-Nitrotyrosine Polyclonal Antibody. The data demonstrate that both the Affinity Sorbent and the Polyclonal Antibody recognize only nitrotyrosine containing proteins.

## Troubleshooting

| Problem | Possible Causes | Recommended Solutions |
|---|---|---|
| No bands seen | Incorrect secondary antibody | Ensure appropriate secondary antibody is used for detection |
| No nitrated proteins | Too little lysate | Add more lysate to matrix |
| Target protein not seen | Low abundance in lysate | Increase amount of sorbent |

## References


1. Beckman, J.S., Chen, J., Ischiropoulos, H., *et al. Methods Enzymol.* **233**, 229-240 (1994).
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3. Beckman, J.S., Ye, Y.Z., Anderson, P.G., *et al. Biol. Chem. Hoppe-Seyler* **375**, 81-88 (1994).
4. Wang, P. and Zweier, J.L. *J. Biol. Chem.* **271**, 29223-29230 (1996).
5. Myatt, L., Rosenfield, R.B., Eis, A.L.W., *et al. Hypertension* **28**, 488-493 (1996).
6. Abe, K., Pan, L.-H., Watanabe, M., *et al. Neurol. Res.* **19**, 124-128 (1997).
7. Chou, S.M., Wang, H.S., and Komai, K. *J. Chem. Neuroanat.* **10**, 249-258 (1996).
8. Ferrante, R.J., Shinobu, L.A., Schulz, J.B., *et al. Ann. Neurol.* **42**, 326-334 (1997).




## NOTES

## Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.