Instruction Manual
for

Nitrotyrosine Assay Kit, Chemiluminescence Detection

Catalog # 17-376 Lot # 30577

Sufficient reagents for 192 assays per kit

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NOT RECOMMENDED OR INTENDED FOR DIAGNOSIS OF DISEASE IN HUMANS.

DO NOT USE IN HUMANS.

I. STORAGE AND STABILITY

Storage: Upon receipt, store individual components at recommended temperatures. Store the white plates at room temperature. Store the Goat Anti-Rabbit IgG, HRP Conjugate (12-348-MN), Anti-Nitrotyrosine, 200X (07-752), and BSA , nitrated (13-127) at -20ºC. If required, aliquot upon receipt to avoid future freeze-thaw cycles. Store all other components at 4ºC.
Stability: Components stable for 6 months from date of shipment if stored and handled correctly.

II. ASSAY OVERVIEW

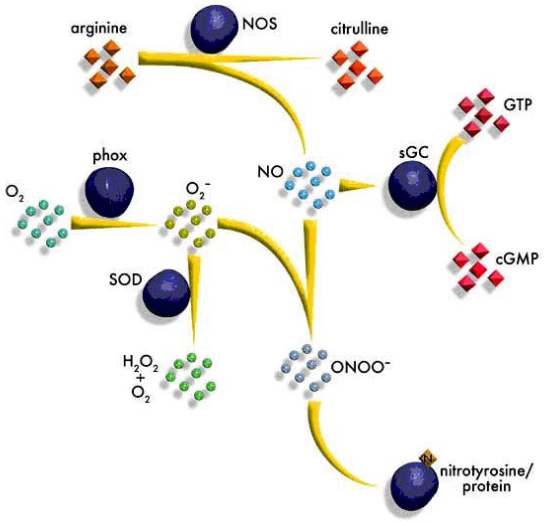
Introduction
Oxidative stress has been implicated in the pathogenesis of neurodegenerative disorders, cancer, and aging. Oxidative stress occurs as a response to increased oxidants, decreased anti-oxidants, or failure to repair oxidative damage induced by reactive oxygen species (ROS). ROS are free radicals, reactive molecules containing oxygen, or molecules containing oxygen that generate free radicals. ROS include nitric oxide (NO), superoxide (O_2^-), peroxynitrite ($ONOO^-$), and hydroxyl radical (OH^-). ROS are cleared from cells by the action of superoxide dismutase (SOD), catalase and peroxidases.

NO, produced by nitric oxide synthases (NOS), is a critical signaling molecule involved in many physiological processes including muscle contraction, vasodilation, neuronal transmission, and immune responses. Some of these effects are mediated through reaction with and activation of guanylate cyclase (GC), resulting in increased cGMP production. With a half-life of seconds, NO readily diffuses through the cytosol and cell membrane, allowing it to act inside or adjacent to the cell in which it was produced.

ROS may interact to form agents that modify cellular proteins. Superoxide, produced by NADPH oxidase (phox), reacts with NO to form peroxynitrite. Intracellular peroxynitrite can modify proteins by interacting with and nitrating tyrosine residues to form 3-nitrotyrosine. Tyrosine nitration may increase (*e.g.* sGC, Src, PI3K, Akt), decrease (*e.g.* Mn-SOD, Ca^{++}-ATPase), or have no discernable effect (*e.g.* p53, VASP, α-Synuclein) on the activity of a particular protein.

The Nitrotyrosine Assay Kit, Chemiluminescence Detection (Cat. # 17-376) is a competitive ELISA for the quantitation of tyrosine nitration. The kit includes all reagents needed for measurement of tyrosine nitration, including white high binding 96-well plates, nitrated BSA standard (a Nitrotyrosine antibody, LumiGLO® chemiluminescent detection substrate, and wash buffers.

The assay has a wide dynamic range and high precision, making this assay a valuable new tool for the study of tyrosine nitration.



III. SYSTEM COMPONENTS

A. Provided Kit Components

White high binding plates
Two Costar 3922 white high-binding 96-well plates. Store at room temperature.

TBS, 20X
Catalog # 20-190C, lot # 32661
One vial containing **100 mL** of 1M Tris, 3M NaCl, pH 7.4. Store at 4ºC.

20% Tween®-20 (v/v)
Catalog # 20-246, lot # 30124
One vial containing **3 mL** of 20% Tween®-20 in sterile, distilled water. Store at 4ºC.

Blocking Buffer 2, 10X
Catalog # 20-316, lot # 30598
One vial containing **7.5 mL** of a proprietary buffer formulation, **10X stock** containing 0.05% Kathon®. Store at 4ºC.

Anti-Nitrotyrosine, 200X
Catalog # 07-752, lot # 30596
One vial containing immuno-affinity purified rabbit IgG in 25mM Tris, pH 7.4, 137mM sodium chloride, 2.7mM potassium chloride, 0.05% Kathon® and 5mg/ml BSA, **200X stock**. Aliquot upon receipt as needed to avoid future freeze-thaw cycles. Store at -20ºC.

BSA, nitrated
Catalog # 13-127, lot # R0605F00003
One vial containing **1 mg in 200µL** of nitrated BSA in 25mM Tris, pH 7.4, 137mM sodium chloride, 2.7mM potassium chloride. The 3-nitrotyrosine content was determined spectrophotometrically (ε_{438} =4300 M^{-1} cm^{-1}) to be 5.6 mol nitrotyrosine per mol BSA. Aliquot upon receipt as needed to avoid future freeze-thaw cycles. Store at -20ºC.

Goat Anti-Rabbit IgG, HRP-conjugate
Catalog # 12-348-MN, lot # 31986A
One vial containing **25 µg** goat anti-rabbit IgG conjugated to horseradish peroxidase, in **25 µL** 0.02M potassium phosphate, 0.15M NaCl, pH 7.2, 10 mg/mL BSA, 0.01% (w/v) gentamicin sulfate before the addition of glycerol to 50%. Liquid at -20°C.

LumiGLO® Chemiluminescent Substrate Reagent A
Catalog # 20-212-c, lot # 31771A
One vial containing **10 mL** of substrate. Store at 4ºC.

LumiGLO® Chemiluminescent Substrate Reagent B
Catalog # 20-212-d, lot # 31772A
One vial containing **10 mL** of substrate. Store at 4ºC.

B. Required Materials Not Provided

- Sample containing nitrated tyrosine
- 50mM carbonate coating buffer (Na_2CO_3/$NaHCO_3$) pH 9.6
- Ice bucket
- Timer
- Microfuge tubes
- Variable volume (5-200µL) pipet + tips
- Reagent troughs for multichannel pipettes
- Microplate incubator
- Microplate sealing film or Parafilm
- 96-well plate reader capable of measuring luminescence

IV. Nitrotyrosine Assay (Chemiluminescence Detection) PROCEDURE

Safety Warnings and Precautions: The Nitrotyrosine Assay kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

A. General Notes

1. During shipment, small volumes of product may accumulate in the vial cap. For maximum recovery of product, centrifuge the vial prior to removing the cap.
2. Plates may be coated with antigen, blocked, washed, sealed and stored for 1 month at 4°C as described in section IV-C I.
3. When performing washes manually, avoid introducing bubbles into the wells when dispensing liquids into the wells, and ensure wells are not overfilled to avoid cross-contamination between wells. Empty wells by inverting the plate over a sink or aspirating the liquid. Blot the plate on absorbent paper to remove excess liquid.
4. A brief rinse, using 1X TBS or water, is recommended prior to the addition of LumiGLO® Chemiluminescent Substrate to remove any traces of the Tween®-20 which may interfere with HRP activity. All traces of moisture should be removed before the addition of LumiGLO® Chemiluminescent Substrate.

B. Preparation of Assay Solutions

Prepare sufficient volume of each solution based on the number of assays to be performed, plus a slight overage to account for pipetting inaccuracies (either 10% extra or one extra assay point is generally sufficient).

Reaction Component Preparation

1. **50mM carbonate coating buffer (Na_2CO_3/$NaHCO_3$) pH 9.6 (not included):** Prepare a 50mM carbonate buffer (Na_2CO_3/$NaHCO_3$) pH 9.6 fresh.
2. **1X Blocking Buffer:** Prepare the 1X blocking buffer by diluting Blocking Buffer 2, 10X (Catalog # 20-316) 10-fold with water (*e.g.,* for 35 mL 1X blocking buffer combine 3.5 mL Blocking Buffer 2, 10X and 31.5 mL water). Each assay point requires 150 µL for the block step, 100 µL for the primary antibody and 100 µL for the secondary antibody. Store on ice.
3. **5 µg/mL nitrated BSA (antigen):** Prepare the nitrated BSA antigen by diluting the BSA, nitrated (Catalog # 13-127) to 5 µg/mL in 50mM carbonate buffer (Na_2CO_3/$NaHCO_3$) pH 9.6 (*e.g.,* for 10 mL of 5 µg/mL nitrated BSA combine 10 µL of BSA, nitrated and 9.99 mL of 50mM carbonate buffer [Na_2CO_3/$NaHCO_3$) pH 9.6]. Each assay point requires 100 µL. Store on ice.
4. **2X nitrated BSA standard (2400 µg/mL):** Prepare the nitrated BSA standard by diluting the BSA, nitrated (Catalog # 13-127) to 2400 µg/mL in 1X blocking buffer (*e.g.,* for 175 µL of 2400 µg/mL nitrated BSA combine 84 µL of BSA, nitrated and 91 µL 1X blocking buffer).
5. **1X TBS:** Prepare 1X TBS by diluting the TBS, 20X (Catalog # 20-190C) 20-fold with water (*e.g.,* for 500 mL of 1X TBS combine 475 mL water and 25 mL TBS, 20X). Store at room temperature.

6. **1X TBS-T:** Prepare 1X TBS-T by diluting the TBS, 20X (Catalog # 20-190C) 20-fold with water containing 0.05% Tween-20™ (*e.g.,* for 200 mL 1X TBS-T combine 10 mL TBS, 20X, 189.5 mL water and 0.5 mL 20% Tween-20™ (Catalog # 20-246). Store at room temperature.
7. **2X Anti-Nitrotyrosine:** Prepare 2X Anti-Nitrotyrosine by diluting the Anti-Nitrotyrosine, 200X (Catalog # 07-752) 100 fold in 1X blocking buffer (*e.g.,* for 5 mL of 2X Anti-Nitrotyrosine combine 50 µL of Anti-Nitrotyrosine, 200X and 4.95 mL 1X blocking buffer). Each assay point requires 50 µL. Store on ice.
8. **2X Test Sample:** Prepare a sufficient volume of test sample in 1X blocking buffer for the number of assay points. Each assay point requires 50 µL. Store on ice.
9. **1X Goat Anti-Rabbit IgG, HRP-conjugate:** Prepare the 1X Anti-Rabbit IgG, HRP-conjugate by diluting the Anti-Rabbit IgG, HRP-conjugate (Catalog # 12-348-mn) 5000 fold in 1X blocking buffer (*e.g.,* for 10 mL of Anti-Rabbit IgG, HRP-conjugate combine 2 µL Anti-Rabbit IgG, HRP-conjugate and 9.998 mL 1X block buffer). Each assay point requires 100 µL. Store on ice.
10. **LumiGLO® Chemiluminescent Substrate:** During the final wash step of the assay protocol prepare the LumiGLO® Chemiluminescent Substrate by combining equal parts of LumiGLO® Chemiluminescent Substrate Reagent A (Catalog # 20-212c) and LumiGLO® Chemiluminescent Substrate Reagent B (Catalog # 20-212d) (*e.g.,* for 10ml of LumiGLO® Chemiluminescent Substrate combine 5 mL of Reagent A and 5ml Reagent B). **Prepare immediately before use**. Each assay point requires 75 µL.

C. Protocol

This assay is a competitive ELISA performed in a microtiter plate. High binding plates are first coated with antigen, nitrated BSA, and blocked. Next, the competitive ELISA is performed using a nitrotyrosine antibody and a test sample. HRP-conjugated Goat anti-Rabbit IgG and LumiGLO® are used for chemiluminescence detection.

I. Prepare the Assay Solutions as required (see Section IV-B).

II. Coat Plate(s) with Antigen

1. Add 100 µL per well of 5 µg/mL nitrated BSA in 50mM carbonate buffer.
2. Incubate the plates for 2 hours at 37°C or overnight at 4°C.
3. Empty wells by inverting the plate(s) over a sink or aspirating the liquid. Wash the plate(s) two times with 1X TBS-T, then soak for 2-3 minutes in 1X TBS. Blot the plate(s) on absorbent paper to remove excess liquid.
4. Add 150 µL per well of 1X blocking buffer.
5. Incubate the plate(s) for 1 hour at 37°C.
6. Empty wells by inverting the plate(s) over a sink or aspirating the liquid. Blot the plate(s) on absorbent paper to remove excess liquid. The plate(s) may be used immediately. If the plate(s) are to be stored at 4°C (for up to 1 month), wash the plate(s) three times with 1X TBS containing 0.05% sodium azide. Blot the plate(s) on absorbent paper to remove excess liquid. Seal the plate(s) with a plate sealer and store at 4°C.

III. Competitive ELISA

1. Add 50 µL of test sample or standard (see Section D) to the appropriate wells.
2. Add 50 µL of 2X Anti-Nitrotyrosine to each well.
3. Incubate the plate(s) at 37°C for 60 minutes.
4. Empty wells by inverting the plate(s) over a sink or aspirating the liquid. Wash the plate(s) once with TBS-T and two to three times with 1X TBS. Empty wells by inverting the plate(s) over a sink or aspirating the liquid. Blot the plate(s) on absorbent paper to remove excess liquid.
5. Add 100 µL per well of 1X Anti-Rabbit IgG, HRP-conjugate.
6. Incubate the plate(s) at 37°C for 60 minutes.
7. Wash the plate(s) two times with 1X TBS-T and twice with TBS. Empty wells by inverting the plate(s) over a sink or aspirating the liquid. Blot the plate(s) on absorbent paper to remove excess liquid.
8. Add 75 µL per well of the freshly prepared LumiGLO® Chemiluminescent Substrate.
9. Incubate the plate(s) at room temperature for 10 minutes.
10. Measure the luminescence as relative light units (RLU) or counts per second (CPS).

D. Standard Curve

Generate a standard curve (0-1200 μg/mL nitrated BSA) using the following procedure. We recommend performing the standard curve in duplicate.

1. Prepare the 2400 μg/mL nitrated BSA standard in 1X blocking buffer as described in Reaction Component Preparation (section IV B step #3).
2. Prepare 3-fold serial dilutions of the 2400 μg/mL nitrated BSA standard in microfuge tubes (*e.g.,* transfer 55 μL of the 2400 μg/mL nitrated BSA standard to 110 μL of 1X blocking buffer, mixing thoroughly before the next transfer. Repeat this process to make successive 3-fold dilutions.) Use 110 μL of 1X blocking buffer in the last tube for the zero standard. Each assay point requires 50 μL as described in the Elisa protocol (section IV C III step #1).

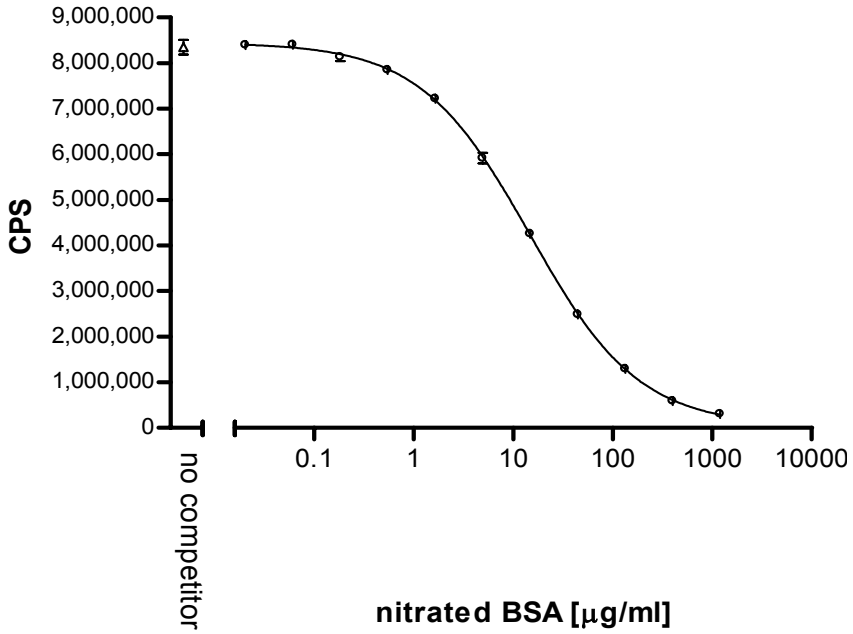


Figure 1. Nitroyrosine Competitive ELISA Standard Curve. The standard curve was performed in triplicate using 1200-0.02 μg/mL nitrated BSA (circles) and 0 μg/mL nitrated BSA (triangle). **NOTE**: This data is presented for reference use only and should not be used to interpret actual assay results. A standard curve must be generated for each assay.

When comparing sample data to standard curve, data should be reported in nitro-BSA equivalents (Kuhn et al, 2004).

V. REFERENCES

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