

Certificate of Analysis

Nitrotyrosine Assay Kit, Chemiluminescence Detection

Catalog # 17-376

Lot # DAM1460106

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 # DAM1460135. One vial containing **100 mL** of 1M Tris, 3M NaCl, pH 7.4. Store at 4°C.

20% Tween®-20 (v/v)

Catalog # 20-246, lot # DAM1460136. One vial containing **3 mL** of 20% Tween®-20 in sterile, distilled water. Store at 4°C.

Blocking Buffer 2, 10X

Catalog # 20-316, lot # DAM1460067. One vial containing **7.5 mL** of a proprietary buffer formulation, **10X stock** containing 0.05% Kathon®. Store at 4°C.

Anti-Nitrotyrosine, 200X

Catalog # 07-752, lot # DAM1460257. 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 -20°C.

BSA, nitrated

Catalog # 13-127, lot # DAM1460066. 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 ($\epsilon_{438} = 4300 \text{ M}^{-1} \text{ cm}^{-1}$) to be 5.6 mol nitrotyrosine per mol BSA. Aliquot upon receipt as needed to avoid future freeze-thaw cycles. Store at -20°C.

Goat Anti-Rabbit IgG, HRP-conjugate

Catalog # 12-348-MN, lot # DAM1394827. 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 # JBC1390548. One vial containing **10 mL** of substrate. Store at 4°C.

LumiGLO® Chemiluminescent Substrate,

Reagent B, Catalog # 20-212-d, lot # JBC1390549. One vial containing **10 mL** of substrate. Store at 4°C.

FOR RESEARCH USE ONLY. NOT RECOMMENDED OR INTENDED
FOR DIAGNOSIS OF DISEASE IN HUMANS
DO NOT USE IN HUMANS

Kit Description

Quantity: 192 assays per kit.

Quality Control: The components of this kit have been matched to optimize sensitivity and range of detection. The kit is used for a competitive ELISA for the quantitation of tyrosine nitration.

Storage and Stability: Components are stable for 6 months from date of shipment when stored at the temperatures indicated above. **Note:** If required, aliquot upon receipt to avoid future freeze-thaw cycles. Store all other components at 4°C.

Use: Read the enclosed protocol before use.

References:

1. Ischiropoulos, H., and Gow, A. (2005) Pathophysiological functions of nitric oxide-mediated protein modifications. *Toxicology*, 208: 299-303.
2. Kharitonov, S.A. (2005) NOS: molecular mechanisms, clinical aspects, therapeutic and monitoring approaches. *Curr. Drug Targets Inflamm. Allergy*, 4: 141-149.
3. Pavher, P., et al. (2005) Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutic strategies. *Curr. Med. Chem.*, 12: 267-275.

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 crosscontamination 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 (Na₂CO₃/NaHCO₃) pH 9.6 (not included):** Prepare a 50mM carbonate buffer (Na₂CO₃/NaHCO₃) 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 (Na₂CO₃/NaHCO₃) 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 [Na₂CO₃/NaHCO₃] 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.

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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 μ L. 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 μ L Anti-Rabbit IgG, HRP-conjugate and 9.998 mL 1X block buffer). Each assay point requires 100 μ L. 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 μ L.

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 μ L per well of 5 μ g/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 μ L 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 μ L of test sample or standard (see Section D) to the appropriate wells.
2. Add 50 μ L 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 μ L 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 $\mu\text{g}/\text{mL}$ nitrated BSA) using the following procedure. We recommend performing the standard curve in duplicate.

1. Prepare the 2400 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$ nitrated BSA standard in microfuge tubes (*e.g.*, transfer 55 μL of the 2400 $\mu\text{g}/\text{mL}$ 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).

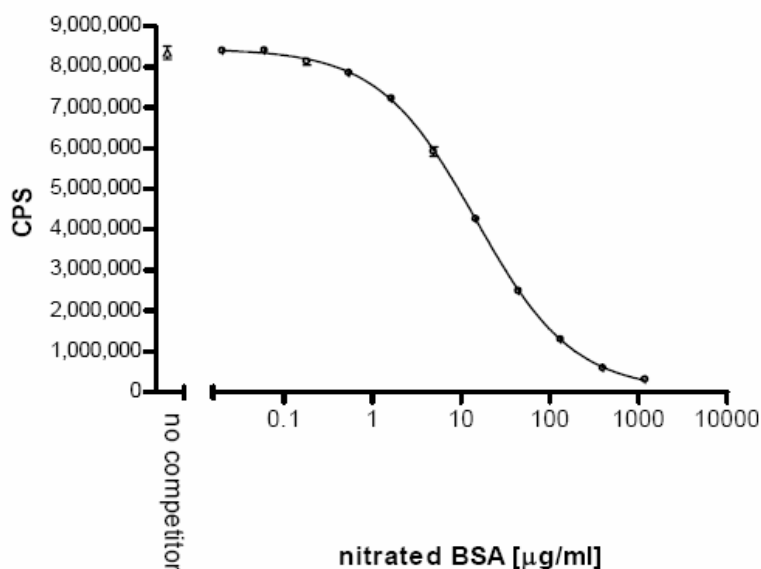


Figure 1. Nitrotyrosine Competitive ELISA Standard Curve. The standard curve was performed in triplicate using 1200-0.02 $\mu\text{g}/\text{mL}$ nitrated BSA (circles) and 0 $\mu\text{g}/\text{mL}$ nitrated BSA (triangle). **NOTE:** This data is presented for reference use only and should not be used 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).

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