Nitrotyrosine Assay Kit

Chemiluminescence Detection

Catalog No. 17-10006

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Introduction

The Nitrotyrosine Assay Kit, Chemiluminescence Detection (Cat. # 17-10006) is a competitive ELISA for the semi-quantitation of tyrosine nitration. The kit includes all reagents needed for measurement of tyrosine nitration, including a white high binding 96-stripwell streptavidin capture plate, single nitrated Tyrosine peptide standard, anti-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.

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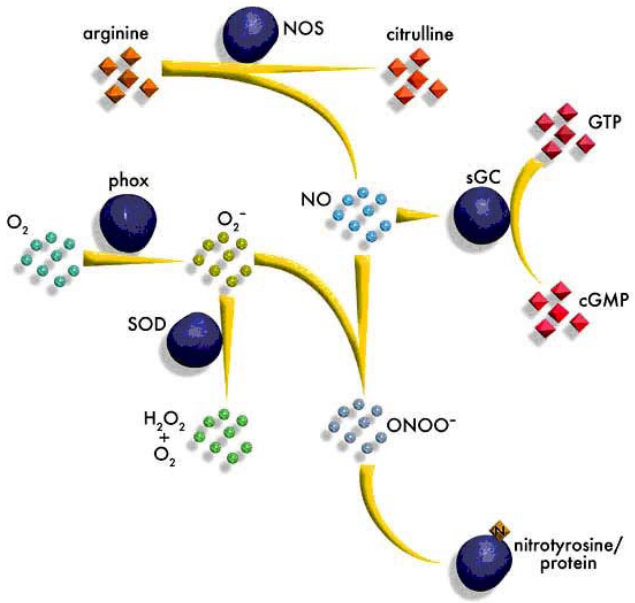
Background

Oxidative stress has been implicated in the pathogenesis of neurodegenerative disorders, cancer and aging. Oxidative stress occurs as a response to increased oxidants, decreased antioxidants, 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 (O2-), 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.



Kit Components

Store at 2-8℃

1. Streptavidin Capture Plate: (Part No. CS204971) One pre-coated 96-stripwell immunoplate sealed in a foil pouch.
2. U-Bottom Plate: (Part No. CS205781) One clear non-binding U-bottom plate.
3. TBS, 20X: (Part No. 20-190) One bottle (50 mL) of 50X TBS.
4. 20% Tween® 20: (Part No. 20-246) One bottle (3 mL) of 20% Tween® 20 (v/v).
5. LumiGlo Chemiluminescent Substrate Reagent A: (Part No. 20-212C) One bottle (10 mL) of substrate.
6. LumiGlo Chemiluminescent Substrate Reagent B: (Part No. 20-212D) One bottle (10 mL) of substrate.
7. Plate Covers: Two plate covers.

Store at -20℃

8. Nitrotyrosine Peptide Standard: (Part No. CS204980). One vial containing (200 µL) of Nitrotyrosine Peptide Standard Stock at 4.8mM.
9. 1000X Biotinylated Nitrotyrosine Peptide: (Part No. CS204990). One vial containing (15 µL) of Biotinylated Nitortyrosine Peptide Stock at 450µM.
10. 2000X Anti-Nitrotyrosine Detection Antibody: (Part No. CS204982) One vial (10 µL) of 2000x anti-Nitrotyrosine detection antibody.
11. Gt X Rb, HRP Secondary Antibody: (Part No. 90276) One vial (30 µL) of 2000X goat anti-rabbit HRP conjugate.
12. 30% BSA: (Part No. CS205797) One vial containing 1mL of 30% BSA.

Materials Not Supplied

1. Multi-channel or repeating pipettes
2. Plate shaker (optional)
3. Pipettors and tips capable of accurately measuring 1-1000 µL
4. Graduated serological pipettes
5. 96-well microtiter plate reader with luminescence readout.
6. Graphing software for plotting data or graph paper for manual plotting of data
7. Microcentrifuge tubes for standard and sample dilutions
8. Mechanical vortex
9. 1 liter container
10. Distilled or deionized water

Storage

Do not use reagents beyond 4 months from date of receipt. Avoid repeated freeze/thaw cycles, aliquot if necessary.

Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and the failure to produce accurate data.
- **Safety Warnings and Precautions:** This 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.
- The Detection Antibody contains sodium azide. Sodium azide may react with copper and lead plumbing to form highly explosive metal azides. Upon disposal, flush with large amounts of water to prevent azide build-up. Avoid contact with skin.

Technical Notes

- All kit reagents should be brought to room temperature (20℃ to 25℃) just prior to use.
- Do not use reagents beyond the expiration date of the kit.
- Do not mix or interchange reagent from various kit lots.
- Manual Plate Washing: Vigorous washing and complete removal of all liquid by aspiration at the end of each washing step is very important to obtain low background values. Gentle agitation during the wash steps or a 2-3 minutes soak may reduce background values.
- The desiccant enclosed in the 96-well capture plate pouch will keep the plate stable when stored at 2° to 8℃ should the pouch lose its seal during shipping.

Samples Preparation

1. Stimulate cultured cells to induce tyrosine nitration.
2. Remove culture media and wash cells twice with ice-cold 1X TBS (Tris Buffered Saline) or PBS (Phosphate Buffered Saline). Discard supernatant.
3. Add 500 µL-1 mL of cold 1X RIPA containing protease inhibitors per 150 mm tissue culture plate.

   Note: 10 mL of 1X RIPA containing protease inhibitors can be prepared by adding 10 µL of 1 µg/µL Leupeptin, 10 µL of 1 µg/µL Aprotinin, 10 µL of 1 µg/µL Pepstatin, 100 µL of 100mM PMSF, 1 mL 10X RIPA (Part No. 20-188) to 8.87 mL of distilled or deionized water.
4. Scrape cells from plate with a cell scraper.
5. Transfer cells in RIPA buffer to a microcentrifuge tube and incubate on ice for 15 minutes.
6. Vortex tube for 10 seconds or sonicate briefly for 10 seconds.
7. Clarify lysate by centrifugation at 12,000 rpm for 10 minutes at 4℃ in a microcentrifuge prior to use.

8. Cell extract containing SDS must be diluted to 0.01% SDS using 1X assay blocking buffer prior to use.
9. Collect the supernatant and calculate protein concentration using a Bradford Assay or by densitometry.
10. It is suggested that the cell lysate be used immediately following preparation. However, samples can be frozen and stored at -80° C for late r use. Frozen samples should be used within 6 months if storing at -80° C. Avoid repeat ed freeze thaws.

Further information regarding lysate preparation protocols can be obtained at *http://www.millipore.com* Cell Lysate Extracts-General Protocols.

Reagents Preparation

1. **Prepare 200mL of 1X TBS.** Combine 10mL TBS, 20X (20-190) with 190 mL Distilled Water.
2. **Prepare 800mL 1X Wash Buffer.** Combine 2.0 mL 20% Tween – 20 (20-246), 40 mL 20X TBS (20-190), 758 mL Distilled Water.
3. **Prepare 30 mL Assay Blocking Buffer.** Combine 1 mL of 30% BSA with 29 mL of 1X wash buffer prepared as above. Store at 4C.
4. **Nitrotyrosine Peptide Standard:**
   i. Label 7 test tubes #2-7 and "0 dose". Add 160 µL of assay blocking buffer to tubes #2-7 and "0 dose".
   ii. The Nitrotyrosine Peptide Standard (Part No. CS204980) is ready to use. This is Standard tube #1 with a concentration of 4800µM (this will be diluted by 3 in assay to result in a 1600uM top point in the standard curve).
   iii. Standards #2-7 are then prepared by performing a 5-fold serial dilution of the preceding standard. For example, to make Standard #2, remove 40 µL of Standard #1 and add it to tube #2 and vortex, and so on. Do not add any Standard to the "0 dose" tube.
5. **Biotinylated Nitrotyrosine Peptide Solution:** Prepare 6 mL per plate of Biotinylated Nitrotyrosine Peptide Solution by diluting 6 µL of 1000x Biotinylated Nitrotyrosine Peptide Stock (450µM) (Part No. CS204990) in 6 mL of assay blocking buffer to result in a 450nM solution (this will be further diluted by 3 in assay to result in a 150nM solution).
6. **Anti-Nitrotyrosine Detection Antibody Solution:** Prepare 6 mL per plate of anti-Nitortyrosine Detection Antibody Solution by diluting 3 µL of 2000x anti-Nitrotyrosine Detection Antibody (Part No. CS204982) in 6 mL assay blocking buffer. Vortex the solution to ensure antibody is evenly diluted in the solution. Discard any unused portion following assay completion. Store at 4℃.
7. **Gt X Rb, HRP Secondary Antibody Solution:** Prepare 12 mL per plate of Secondary Antibody Solution by diluting 6 µL of Gt X Rb, HRP Secondary Antibody (Part No. 90276) in 12 mL assay blocking buffer. Vortex the solution to ensure antibody is evenly diluted in the solution. Discard any unused portion following assay completion. Store at 4℃.
8. **LumiGlo Chemiluminescent Substrate Solution**: During final wash step of the assay protocol, prepare the LumiGlo Chemiluminescent Substrate Solution by combining equal parts of LumiGlo Reagent A (Part No. 20-212C) and LumiGlo Reagent B (Part No. 20-212D). For

example, for 12 mL of LumiGlo Chemiluminescent Substrate combine 6 mL of Reagent A and 6 mL of Reagent B). **Prepare immediately before use**. Each assay point requires 100 µL.

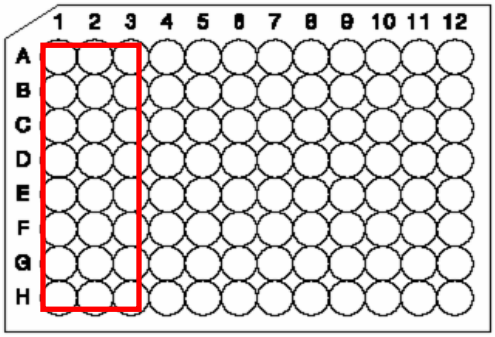
Assay Protocol

1. Take out 3 strips to perform the test. Store the remaining strips with descant at 4℃.

Strips Layout:

STD (uM)

1. 1600
2. 320
3. 64
4. 12.8
5. 2.56
6. 0.512
7. 0.1024
8. 0



2. In U-bottom plate (Part No. CS205781), setup wells from A1 to H3 with Nitrotyrosine Peptide Standard. Add 40 µL/well of the standard solution prepared in step 4 above.
3. Add 40µL/well of the Biotinylated Nitrotyrosine Peptide Solution prepared in step 5 above to U-bottom plate containing peptide standard. Mix by pipetting up and down.
4. Add 40 µL per well of the anti-Nitrotyrosine Detection Antibody Solution prepared in step 6 above. Mix by pipetting up and down. Seal the strips and incubate at room temperature for 1 hour with mild agitation.
5. Transfer 100µL/well of mixture from U-bottom plate to Streptavidin coated plate (Part No. CS204971). Seal the strips and incubate at room temperature for 1 hour with mild agitation. Discard U-bottom plate and remaining solution.
6. Remove the mixture with a wrist flick and wash the wells with 200 µL per well of 1X TBST two times quickly, followed by two times with 5 minutes each of gentle agitation. When washing is finished, tap the plate gently over a stack of absorbent papers to remove excess liquid.
7. Add 100 µL per well of the Secondary Antibody Solution prepared in step 7 above. Seal the plate and incubate at room temperature for 1 hour with mild agitation.

Note: Bring LumiGlo Reagent A (Catalog 20-212C) and LumiGlo Reagent B (20-212D) to warm up at RT.

8. Remove Secondary Antibody Solution with a wrist flick and wash the wells with 200 µL per well of 1x TBST two times quickly, followed by two times with 1X TBS quickly. Then wash two times with 1x TBS with 5 minutes each with mild agitation. When washing is finished, tap the plate gently over a stack of absorbent paper to remove excess liquid.
9. Add 100 µL per well of the LumiGlo Chemiluminescent Substrate Solution prepared in step 8. Incubate in dark for 5 minutes.

10. Measure the luminescence as counts per second (CPS).

CAUTION: *Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the reading.*

NOTE: *For very low starting protein levels, samples can be placed at 37°C during the final incubation to obtain greater sensitivity.*

Assay Results

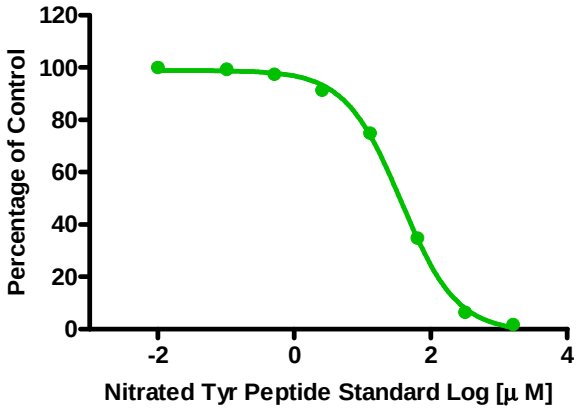


Figure 1. Typical Nitrotyrosine Standard Curve
40 µL of progressive 5-fold dilutions of the Nitrotyrosine Peptide Standard included in the kit and run as described in the assay instructions.

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.

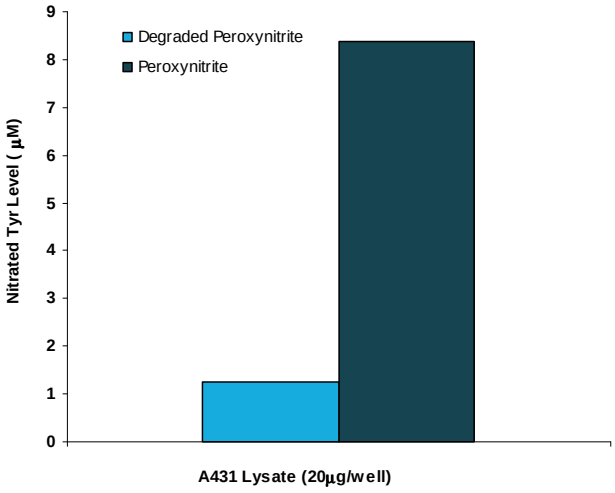


Figure 2. Cell lysate validation with 17-10006 Nitrotyrosine Kit
A431 cells were treated with 5mM degraded or non-degraded peroxynitrite for 5 minutes; a total 20 µg /well protein were applied following the assay protocol. The peroxynitrite treated cells showed an enhanced nitrotyrosine level over the degraded peroxynitrite treated cells.

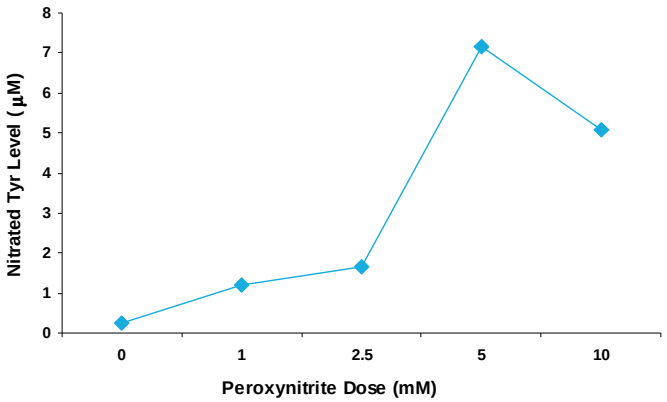


Figure 3. Dose response of peroxynitrate to A431 cells.
A431 cells were treated with various concentrations of peroxynitrite for 5 minutes; a total of 20 µg lysate per well were applied following the assay protocol. The data shows an increasing level of nitrotyrosine as the increase in peroxynitrite concentration, peaked at 5 mM, then dropped at 10 mM.

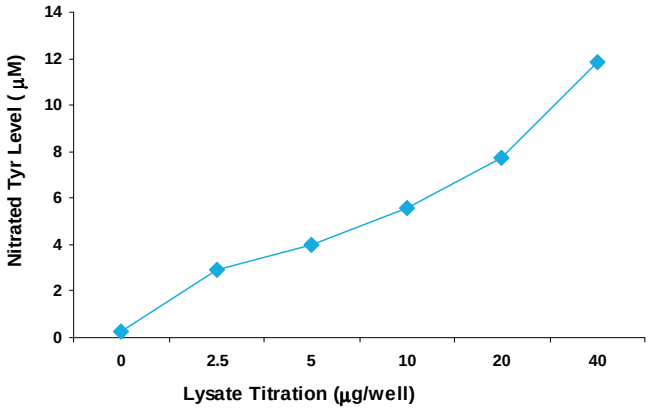


Figure 4. Lysate titration of 5mM peroxynitrite treated A431 cells.
A431 cells were treated with 5 mM peroxynitrite for 5 minutes; various amount of total lysate were applied following the assay protocol. The data shows that the assay detects as low as a 2.5 µg lysate per well and the nitrotyrosine level increased as the lysate amounts applied increased.

Troubleshooting Guide

Problem	Potential Cause	Experimental Suggestions
No signal or weak signal in all wells	Missing components or key steps	Check to make sure all components were added in the appropriate steps and amounts.
	Standard or Biotinylated-Peptide or Capture plate is no longer active or has reduced activity	Make sure all components are stored at the recommended temperature and minimize the freeze/thaw cycle as manual recommends. Make aliquots of components when first thawed if planning more than one assay.
	Plate reader or settings are not optimal	Verify the measurement, read time, and filter on the plate reader.
	Incorrect storage temperatures Incorrect assay temperature	Items are to be stored at the appropriate storage temperatures. Performance can be negatively affected if reagents are not stored and used in the appropriate time period.
No detectable signal in samples	Low nitrotyrosine level or out of the standard curve range or poor lysate preparation.	Check the enclosed sample preparation protocol for instructions for sample preparation, make a proper dilution if out of detectable range. Prepare a SDS PAGE gel and blot of test samples, apply the specific anti-nitrotyrosine antibody 1:2000 to perform a western blot to check the sample nitration.
Too strong signal in sample wells	High nitrotyrosine level.	Make a serial dilution of test samples, adjust for a proper dilution fitting into the standard curve range.

References

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