# Nitric Oxide Cell-Based HTS Assay Kit (#BN01137)

**(Catalog # BN01137; 100 assays; Store at -20°C)**

## I. Introduction:

Nitric oxide (NO) plays an important role in neurotransmission, vascular regulation, immune response and apoptosis. NO has been established as one of the key regulators in cardiovascular, nervous, and immune systems. NO plays a pivotal role in numerous processes: in central nervous system, NO participates in cell communication and information storage; in vascular endothelium, NO is involved with regulation of vascular function. Finally, NO is produced by immune cells, including macrophages, as a part of body's defense mechanism during immunological responses and oxidative stress conditions. However, chronic overproduction of NO is one of the fundamental causes underlying disorders including neurodegenerative diseases and pathophysiology of blood vessels. Assay Genie's Nitric Oxide Cell-Based HTS Assay Kit utilizes a dye that reacts with intracellular NO to produce a highly-Fluorescent Triazole Product (Ex/Em= 488/532 nm). The kit includes Diphenyleneiodonium (DPI), a potent inhibitor of nitric oxide cellular production, which has been widely used to inhibit NO synthesis in order to evaluate NO function in different systems and diseases. This kit provides a simple, standardized and HTS method to quantitate the amount of NO in cell-based assays. This Assay Kit is user-friendly, sensitive and can detect the NO Fluorescent product as low as 5 pmol in a 96-well plate.


## II. Application:

- High-Throughput quantitative measurement of Nitric Oxide production.
- Screening/Characterization of stimulators/ inhibitors of Nitric Oxide and NOS in cells.

## III. Sample Type:

- Nitric Oxide-producing cells
- Adherent or suspension cells

## IV. Kit Contents:

| Components | BN01137 | Cap Code | Part Number |
|---|---|---|---|
| NO Assay Buffer | 50 ml | NM | BN01137-1 |
| NO Staining Dye | 20 µl | Brown | BN01137-2 |
| NO Inhibitor (DPI, 1mM) | 20 µl | Orange | BN01137-3 |
| NO Standard (100 µM) | 25 µl | Yellow | BN01137-4 |

## V. User Supplied Reagents and Equipment:

- 96-well plates (sterile, white or black plate with clear flat bottom)
- Multi-well spectrophotometer (Ex/Em = 488/532 nm)
- NO-producing primary cells or cell lines
- Cell-Culture Media
- 37°C Incubator with 5% $CO_2$

## VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C. Read entire protocol before performing the assay. Open all reagents under sterile conditions (e.g. cell culture hood).

- **NO Assay Buffer:** Store at 4°C or -20°C. Warm to 37°C before use. Use within 6 months.
- **NO Staining Dye** and **NO Standard:** Light sensitive. Do not expose to direct-intense light. Aliquot and store at -20°C. Avoid multiple freeze/thaw. Use within 6 months.
- **NO Inhibitor (DPI):** Aliquot and store at -20°C. Avoid multiple freeze/thaw. Use within 6 months.

## VII. Nitric Oxide Quantitative Measurement Protocol:

1. **Cell Culture:** Grow cells in appropriate media and culture conditions. Adherent cells should be cultured to ~80% confluency. For both adherent and suspension cells, harvest cells and centrifuge at 1,000 x g, for 5 min. Resuspend the cell pellet in NO Assay Buffer and count the number of cells using a hemocytometer or an automated cell counter. Re-suspend cells in 1 ml of media at the concentration of 5 x $10^6$ cells/ml. For 96 well-plate, add 1 ml of re-suspended cells to 9 ml of media. Mix gently by pipetting, and add 100 µl of cell mixture to each well to get 50,000 cells per well.

   **Note:**

   **a.** For cells in suspension, after initial seeding, it is important to centrifuge the entire plate at 1,000 x g for 5 min, every time, before removing, replacing with any media or solutions.

   **b.** Prepare at least 1 blank well without cells or media for background control (Dye-only) assessment (see Step **VII-3b**)

2. **Inhibitor Control and Test Compound Preparation:**
   - **a.** For Inhibitor Control (**IC**), add 4 µl of DPI Inhibitor stock (1mM) to 500 µl of pre-warmed media concentration (1:125 dilution). Add 100 µl of diluted DPI mixture to 100 µl of pre-existing media in each well to make 200 µl total volume (4 µM DPI final concentration).
   - **b.** For Test Compounds (**TC**) and Vehicle Solvent Control (**SC**), dissolve test compounds in proper solvent(s) to produce 100X stock solutions. To make a master mix (for every 5 wells), add 12 µl of each test compound stock (100X) or 12 µl of Vehicle Solvent to 588 µl of media. Add 100 µl of media mixture with Test Compounds or Solvent to well to make to make 200 µl total volume (1x TC final concentration).
   - **c.** Pre-incubate the compounds with cells for 1 h at 37°C.

   **Note:**

   - **i.** Pre-incubation time with inhibitors or activators may vary, depending on the test compounds.
   - **ii.** Final solvent concentration should be minimized to avoid solvent toxicity to cells. DMSO has been shown to have negligible effects on cell viability at a concentration of ≤ 2%.
3. **Background Controls (BC) Preparation:** prepare the following **BC** conditions *(for calculations, choose the higher RFU measurement between them as your background)*:
   - **a.** Cell-only control (**CC**) well: Add 100 µl of fresh media to 100 µl pre-existing media in each well to make 200 µl total volume.
   - **b.** Dye-only control (**DC**) well: make sure the well is empty and without cells or media. Proceed to next step.
4. **Standard curve**: Add 5 µl of the NO Standard stock solution (100 µM) to 95 µl NO assay buffer to generate 5 µM working solution. Add 0, 2, 4, 6, 8, 10 µl of the diluted Standard to 6 consecutive blank wells (without cells) to generate 0, 10, 20, 30, 40, 50 pmol/well respectively. Bring the volume of each well to 50 µl with Assay Buffer.
5. **Staining dye:**
   - a. Pre-warm NO Assay buffer to 37°C. To make a master mix, for every 25 wells, add 3 µl of Staining Dye Stock to 1.5 ml of Assay Buffer (1:500 dilution).
   - b. Remove the media from each well by vacuum aspiration, and add 50 µl of Staining Dye diluted solution to wells containing **IC**, **TC**, **SC and DC**. For **CC**: Add 50 µl of NO Assay Buffer without staining dye.
   - c. Incubate the entire plate for 1 h at 37°C in the dark. No additional wash is needed after this step. The plate is ready for measurement after incubation.
6. **Measurement**: Measure fluorescence (RFU) by plate reader (Ex/Em = 488/532 nm). Top-read setting is recommended.
7. **Calculation:** For Standard Curve, subtract 0 Standard reading from all readings and plot the Standard Curve. For samples, subtract the background control reading (from **step VII-3)** from all sample readings. Apply ΔRFU to the Standard curve to get the amount of NO (pmoles) generated during the reaction. **Note:** 1 pmol of Fluorescent Triazole= 1 pmol of NO.

**B = Nitric Oxide (pmol) = [ΔRFU (TC - BC) ] / Slope of Std. Curve**

Where: **TC:** test compound, **BC:** Background Control

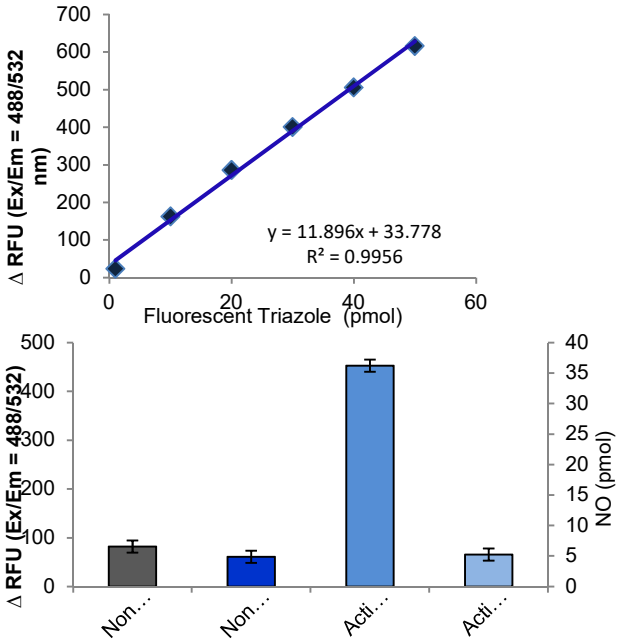


**Figure 1: (a)** Standard curve of Fluorescent Triazole product (pmol). Standard 0 reading was subtracted from all readings. **(b)** Macrophage cells (J774A.1) were activated with LPS (200 ng/ml) and γ-IFN (100 ng/ml) for 24 hrs (orange & green bars respectively). The following day, a subset of wells containing activated and non-activated cells were treated with NO inhibitor (DPI 4 µM, blue & green) or vehicle control (0.5% DMSO, grey & orange respectively) for 1 h. After media removal, cells were stained with the NO Staining Dye for 1 h at 37°C, and measurements were taken using spectrophotometer (Ex/Em = 488/532 nm). Fluorescent measurement demonstrated that activated-macrophages produce higher NO levels, that is inhibited by DPI (green bar) to level of non-activated cells. Fluorescent Δ RFU was converted to pmol of NO product using equation provided. **Note:** Background control (**CC**, in this case), was subtracted from ΔRFU reading. *LPS and γ-IFN reagents are not included in kit.*

***FOR RESEARCH USE ONLY! Not to be used on humans***