Cell Meter™ Fluorimetric Intracellular Nitric Oxide Assay Kit

NIR Fluorescence Optimized for Microplate Reader

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 16359 (200 assays)	Keep in freezer, protect from light	Microplate Reader Fluorescence Microscope

Introduction

Nitric oxide (NO) is an important biological regulator involved in numbers of physiological and pathological processes. Altered NO production is implicated in various immunological, cardiovascular, neurodegenerative and inflammatory diseases. As a free radical, NO is rapidly oxidized and there is relatively low concentrations of NO existing in vivo. It has been challenging to detect and understand the role of NO in biological systems. Cell Meter™ Fluorimetric Intracellular Nitric Oxide Assay Kits provide sensitive tools to monitor intracellular NO level in live cells. Nitrixyte™ probes are developed and used in our kits as an excellent replacement for DAF-2 for the detection and imaging of free NO in cells. Compared to the commonly used DAF-2 probe, Nitrixyte™ probes have better photostability and enhanced cell permeability. This particular kit uses Nitrixyte™ NIR that can react with NO to generate strong near-infrared (NIR) fluorescence signal. Nitrixyte™ NIR can be readily loaded into live cells, and its fluorescence signal can be conveniently monitored using the filter set of Cy5® or APC. This kit is optimized for fluorescence imaging and microplate reader applications.

Kit Components

Components	Amount
Component A: Nitrixyte™ NIR	50 µL (500X)
Component B: Assay Buffer I	1 bottle (20 mL)
Component C: Assay Buffer II	1 bottle (20 mL)

Assay Protocol for Plate Reader

Brief Summary

Prepare cells in growth medium → Incubate cells with test compounds and Nitrixyte™ NIR working solution → Add Assay Buffer II → Monitor fluorescence intensity at Ex/Em = 650/680 nm

1. Prepare cells:

1.1. For adherent cells: Plate cells overnight in growth medium at 30,000 to 80,000 cells/well/90 µL for a 96-well plate or 8,0 00 to 20,000 cells/well/20 µL for a 384-well plate.

1.2. For non-adherent cells: Centrifuge the cells from the culture medium and suspend the cell pellets in culture medium at 125,000-250,000 cells/well/90 µL for a 96-well poly-D lysine plate or 30,000-60,000 cells/well/20 µL for a 384- well poly-D lysine plate. Centrifuge the plate at 800 rpm for 2 minutes with brake off prior to your experiment.
Note: Each cell line should be evaluated on an individual basis to determine the optimal cell density.

2. Prepare working solution:

2.1 Thaw all the kit component at room temperature before use.

2.2 Make Nitrixyte™ NIR working solution for one cell plate: Add 20 µL of Nitrixyte™ NIR stock solution (Component A) into 10 mL of Assay Buffer I (Component B), and mix well. The working solution is stable for at least 2 hours at room temperature.
Note: 20 µL of Nitrixyte™ NIR stock solution is enough for one plate. Aliquoted and stored unused Nitrixyte™ NIR stock solution at ≤ -20 °C. Protect it from light and avoid repeated freeze-thaw cycles.

3. Run the NO assay:

3.1 To stimulate endogenous NO, treat cells with10 µL of 10X test compounds (96-well plate) or 5 µL of 5X test compounds (384-well plate) in cell culture medium or your desired buffer (such as PBS or HHBS). For control wells (untreated cells), add the corresponding amount of medium or compound buffer.
Note: It is not necessary to wash cells before adding compound. However, if tested compounds are serum sensitive, growth medium and serum factors can be aspirated away before adding compounds. Add 90 µL/well (96-well plate)

and 20 µL/well (384-well plate) of 1X Hank's salt solution and 20 mM Hepes buffer (HHBS) or the buffer of your choice after aspiration. Alternatively, cells can be grown in serum-free media.

3.2 Add 100 µL/well (96-well plate) or 25µL/well (384-well plate) of Nitrixyte™ NIR working solution (from Step 2.2) in the cell plate. Co-incubate cells with test compound and Nitrixyte™ NIR working solution at 37 °C for desired period of time, protected from light.
Note 1: DO NOT remove the test compounds.
Note 2: For a NONOate positive control treatment: Cells were incubated with Nitrixyte™ NIR working solution at 37 °C for 30 minutes. The working solution was removed and cells were further incubated with 1 mM DEA/NONOate at 37 °C for 30 minutes to generate nitric oxide. See Figure 1 for details.
Note 3: We have used Raw 264.7 cells incubated with 0.5X Nitrixyte™ NIR, 20 µg/mL of lipopolysaccharide (LPS) and 1 mM L-Arginine (L-Arg) in cell culture medium at 37 ºC for 16 hours. See Figure 2 for details.

3.3 Remove solution in each well. Add Assay Buffer II (Component C) 100 µL/well for a 96-well plate or 25 µL/well for a 384-well plate.
Note: DO NOT wash cells before adding Assay Buffer II.

3.4 Monitor the fluorescence increase using microplate reader at Ex/Em = 650/680 nm (cut off = 665 nm) with bottom read mode, or take images using fluorescence microscope with a Cy5® filter.

Data Analysis

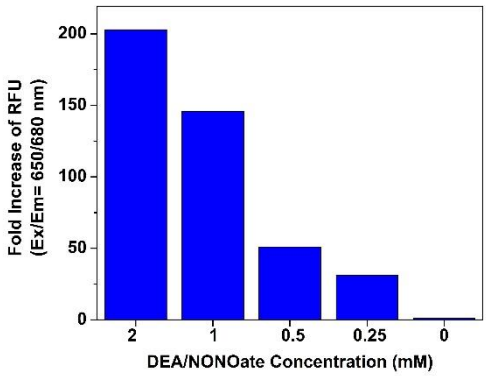

Figure 1. Detection of exogenous nitric oxide (NO) in cells upon DEA/NONOate treatment (NO donor) using Cell Meter™ Fluorimetric Intracellular Nitric Oxide Activity Assay Kit (Cat#16359). CHO-K1 cells were incubated with Nitrixyte™ NIR working solution at 37 ºC for 30 minutes. The working solution was removed to stop the staining. The cells were further treated with or without DEA/NONOate at various concentration (0.25-2 mM) in HBSS with 1 mM HEPES (pH=6.2) buffer at 37 ºC for 30 minutes. The solution in each well was removed, and Assay Buffer II was added before fluorescence measurement. The fluorescence signal was monitored at Ex/Em = 650/680 nm (cut off = 665 nm) with bottom read mode using a FlexStation microplate reader.

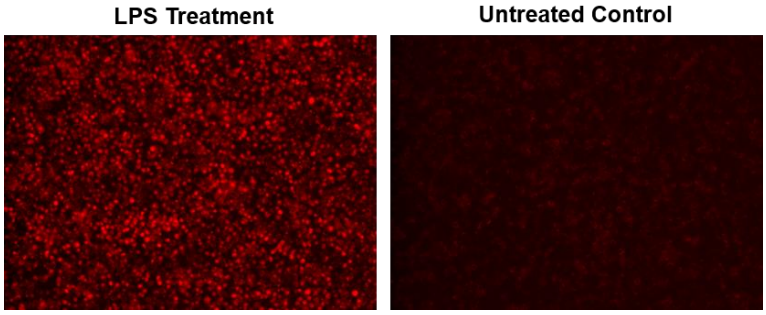

Figure 2. Fluorescence images of endogenous nitric oxide (NO) measurement in RAW 264.7 macrophage cells using Cell Meter™ Fluorimetric Intracellular Nitric Oxide Activity Assay Kit (Cat#16359). Raw 264.7 cells at 100,000 cells/well/100 µL were seeded overnight in a Costar black wall/clear bottom 96-well plate. Cells were co-incubated with Nitrixyte™ NIR, with or without 20 µg/mL of lipopolysaccharide (LPS) and 1 mM L-Arginine (L-Arg) in cell culture medium at 37 ºC for 16 hours. The solution in each well was removed, and Assay Buffer II was added before fluorescence measurement. The fluorescence signal was measured using fluorescence microscope with a Cy5® filter.

References

1. Rose MJ and Mascharak PK. (2008) Fiat Lux: selective delivery of high flux of nitric oxide (NO) to biological targets using photoactive metal nitrosyls. Curr Opin Chem Biol.
2. Mayer B. (2000) Nitic Oxide; Handbook of Experimental Pharmacology. Springer, Berlin.