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SIGMA

ProductInformation

Nitric Oxide Synthase Detection System, Fluorimetric

Catalog Number **FCANOS1**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

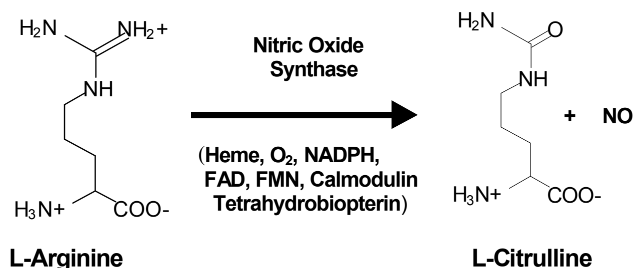
Product Description

Sigma's Fluorimetric Cell-Associated Nitric Oxide Synthase (NOS) Detection System is a simple and specific assay for the measurement of free nitric oxide (NO) and nitric oxide synthase (NOS) activity in living cells under physiological conditions. Nitric oxide, formed from the amino acid L-arginine via the action of NOS, is a unique cell signaling molecule, that functions as both an intracellular and an extracellular messenger. NO has been implicated in vasodilation, cytotoxicity, neurotransmission, and inflammation.[1-3] Many effects of NO are mediated by its ability to activate the enzyme guanylyl cyclase. The resulting increase in intracellular cyclic GMP levels regulates protein kinase G activity, protein phosphorylation, and many biological processes.[4] In contrast, the cytotoxic and inflammatory effects of NO are independent of cyclic GMP and may result from its interaction with metal ions, thiol groups, and other free radicals that, in turn, can mutate DNA and inhibit several key enzymes involved in energy metabolism.[5]

Three distinct isoforms of NOS have been identified, referred to as neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). Moreover, splice variants have been shown to exist for all of these isoforms. NO formed by nNOS (also known as cNOS, bNOS, NOS-1, or Type 1 NOS) in the central nervous system may be important in information storage associated with learning and memory. In contrast, NO synthesized by iNOS (also known as NOS-2 or Type II NOS) may participate in antimicrobial activity, cytotoxicity, and/or inflammatory responses. Lastly, NO formed by eNOS (also known as NOS-3 or Type III NOS) in endothelial cells is responsible for blood pressure regulation by endothelial-dependent vasodilators. Most cells and tissues possess one or more isoforms of NOS. The endothelial and neuronal isoforms are constitutive and highly regulated by Ca^{2+} and calmodulin. The inducible isoform of NOS is expressed *de novo* after exposure to bacterial endotoxins and/or any one of several inflammatory cytokines.

NOS uses arginine as a substrate and converts it to citrulline with the generation of NO.[6,7] This reaction involves a five-electron oxidation of the guanidine nitrogen of L-arginine with molecular oxygen to give the stoichiometric production of NO and L-citrulline (see Figure 1).

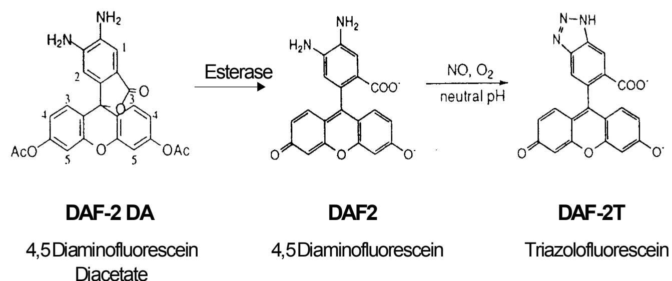
Figure 1.
Biochemical conversion of L-arginine to L-citrulline



In cell-free conditions *in vitro*, the purified NOS enzyme requires the addition of five cofactors ($FMNH_2$, FADH, NADPH, calmodulin, and tetrahydrobiopterin [BH_4]) and two divalent cations (calcium and heme iron). One standard assay for NOS activity in solubilized, cell free enzyme preparations such as cell lysates, crude tissue extracts, or purified enzymes, measures the production of radiolabeled citrulline from radiolabeled arginine substrate.[6]

In contrast, this kit measures the intracellular production of NO by a non-radiometric method. The system utilizes a cell-permeable diacetate derivative of 4,5-diaminofluorescein (DAF-2 DA).[8, 9] DAF-2 DA penetrates cells rapidly, where it is hydrolyzed by intracellular esterase activity to DAF-2 that, in turn, reacts with NO produced by NOS to form a fluorescent triazolofluorescein (see Figure 2).

Figure 2.
Intracellular formation of 4,5-diaminofluorescein, a fluorimetric indicator of nitric oxide or nitric oxide synthase activity



The fluorescent product can be measured using an excitation wavelength of 450–495 nm and an emission wavelength of 505–550 nm. In a model cell system, the murine macrophage cell line RAW 264.7, in which iNOS is induced by lipopolysaccharides (LPS) and γ-interferon (γIFN), it could be demonstrated that the fluorescent signal generated by this compound is specific for iNOS activity.

Reagents
The kit is sufficient for 200 assays in 96 well plates.

Reaction Buffer Catalog Number R2525	50 mL
β-NAD Phosphate Reduced, Tetrasodium (β-NADPH) Catalog Number N9910	2 vials
Arginine Substrate Solution Catalog Number A4344	2 mL
Diphenyleneiodonium Chloride (DPI) 1 mM solution Catalog Number D1688	200 μL
4,5-Diaminofluorescein Diacetate (DAF-2 DA) 5 mM solution in DMSO Catalog Number D225	60 μL

Reagents and Equipment Required but Not Provided
- Nitric Oxide Synthase (NOS) producing cells or cell line.
- Black, flat, clear bottom, 96 well tissue culture plates (Catalog Number CLS3603 or equivalent)
- General tissue culture supplies and media
- Adjustable volume multichannel pipettes or electronic digital repeating pipette
- Spectrofluorometer for 96 well plate

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Preparation of Culture in a 96 Well Plate
This assay system is designed to detect the production of NO via NOS enzymatic activity in live cells under physiological conditions, e.g., using an endothelial, neuronal, or macrophage cell line. These cell lines, in general, have an adherent growth phenotype.
1. Release cells from a culture flask (flasks) by trypsinization or scraping.
2. Centrifuge the cells at 125 × *g* for 10 minutes and dilute with fresh growth medium. Determine the cell density of the suspension and adjusted to 500,000 cells/mL.
3. Culture 200 μl per well, i.e., 100,000 cells per well in a black, clear bottom, 96 well plate. The optimal cell number per well is dependent on the cell size and the amount of NOS expressed per cell. This must be determined empirically for each cell system.

Induction protocol for iNOS in RAW 264.7 machrophage cell line
RAW 264.7 cells can be induced with mouse γ-interferon (Catalog Number I4777, 10 ng/mL final) and LPS (Catalog Number L3129, 50 ng/mL final). The γ-interferon and LPS can be added immediately after cell plating or directly into the cell suspension before plating. Leave a few wells with non-induced cells as negative controls. Induction of iNOS can be detected after 4 hours. The optimal induction duration is 15–24 hours. A cytokine induction protocol for iNOS expression in RAW264.7 macrophage cell line has been published.[10]

Reconstitution of β-NADPH - Single use β-NADPH vials are provided with the kit for two β-NADPH titration experiments. Before use, add 331 μL of 18 MΩ·cm or equivalent water to the vial and vortex for a few seconds to obtain a 1 mM β-NADPH solution. Keep on ice before use.

Storage/Stability
The kit ships on wet ice and storage at 2–8 °C is recommended.

Procedure
Perform the assay reactions in duplicates

It is recommended to include the following controls in the experimental design:

a. Negative biological control: non-induced cells
b. Cell-free baseline: complete reaction mix without cells for the initial determination of baseline fluorescence.
c. Isoform specific inhibition. A potent inhibitor of inducible NOS (iNOS), diphenyleneiodonium chloride (DPI) is supplied with the kit. DPI inhibits iNOS in RAW 264.7 cells completely at 1-2 μM with an IC_{50} of 100 nM. We recommended establishing the effective range for every cell system starting with a range of 0.125–8 μM.
d. Minus dye control for background fluorescence.

Under some conditions, depending on the cell line and growth conditions, addition of β-NADPH to the reaction mixture may enhance NOS activity and therefore, may also enhance the signal/noise ratio. The optimal β-NADPH concentration should be carefully titrated. It is recommended to start the titration in the range of 0.5–4 μM final β-NADPH concentration. The most effective β-NADPH concentration may be used in following experiments. The β-NADPH vial is for single use only. Discard any remaining 1 mM β-NADPH solution after use.

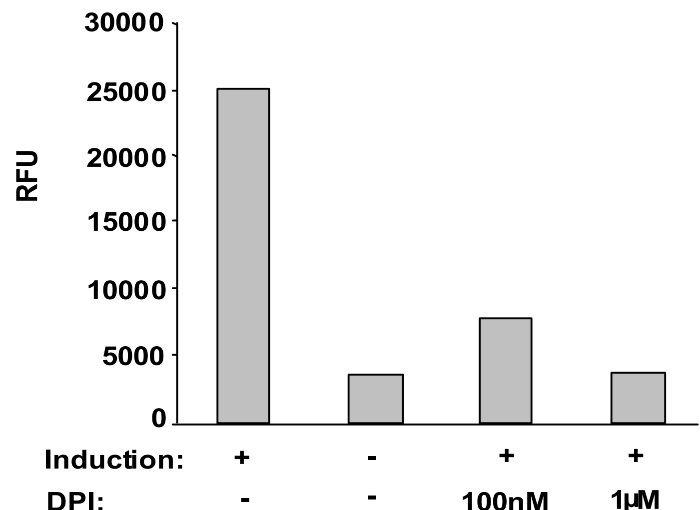
Assay Protocol

1. Set the fluorimeter at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Note: Triazolofluorescein can be detected using an excitation wavelength range of 450-495 nm and an emission wavelength range of 505-550 nm.
2. Prepare appropriate reaction mixtures. For each assay in the 96 well plate, prepare 200 μL of reaction mixture. Table 1 indicates the volume of each reagent required for a single assay. For multiple assays calculate accordingly.
3. Remove the growth medium from the wells.
4. Dispense 200 μL of the appropriate reaction mixture into each well.
5. Incubate for 2 hours at room temperature in the dark.
6. Read the fluorescence

Table 1.
Reaction Mixtures

Reaction	**Arginine Substrate Solution** (A4344)	**DAF-2 DA Solution** (D225)	**DPI or NADPH Solution**	**Reaction Buffer** (R2525)
NOS Assay	10 μL	0.1 μL	–	190 μL
Blank - no cells	10 μL	0.1 μL	–	190 μL
Control - non-induced cells	10 μL	0.1 μL	–	190 μL
Control - minus dye	10 μL	–	–	190 μL
Inhibition reaction (optional)	10 μL	0.1 μL	x μL	190–x μL
NOS Assay + NADPH (optional)	10 μL	0.1 μL	x μL	190–x μL

Results
Figure 3.
Induction of Nitric Oxide Synthase (NOS) activity in RAW 264.7 cells



RAW 264.7 cells were incubated for 20 hours with LPS and mouse γ-interferon prior to the NOS activity measurement. The results shown are of the fluorescence detected after a 2 hour reaction.
Excitation wavelength – 485 nm
Emission wavelength – 530 nm
RFU = Relative Fluorescence Units

Troubleshooting Guide

Problem	Cause	Solution
No significant difference between induced and non-induced cells	No induction	Try other inducers.
A difference between induced and non-induced cells is observed, but is not significant.	Cell density is too low.	Plate more cells.
DPI has little or no effect.	No induction	Try other inducers.
Very high background	Overlapping spectrum pass between the excitation and emission filters	Try another filter combination.
	White plate or clear plate is used.	Try to use black, flat, clear bottom plate.

References

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KAA, LPG, EB, MAM 01/07-1

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