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ab211085 Intracellular Nitric Oxide Synthase Detection Assay Kit

For the rapid, sensitive detection of intracellular Nitric Oxide Synthase (NOS) in live cells.

This product is for research use only and is not intended for diagnostic use.

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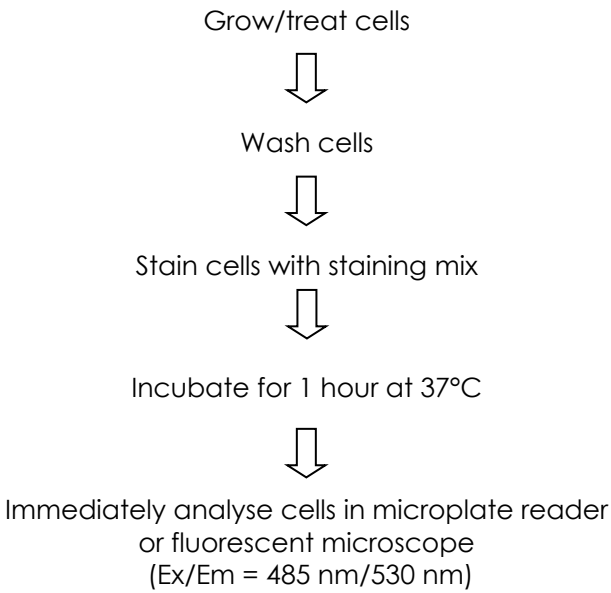
1. Overview

Intracellular Nitric Oxide Synthase Detection Assay Kit (ab211085) provides a simple, non-radiometric method for detection of intracellular nitric oxide synthase (NOS) in cells. The kit uses a dye that reacts with intracellular nitric oxide (NO) produced by the cellular NOS to produce fluorescence (Ex/Em = 485/530 nm), which is proportional to the concentration of intracellular NOS. Fluorescence can be detected using a microplate reader or a fluorescence microscope.

Nitric oxide synthases (EC 1.14.13.39) (NOS) are a family of enzymes that catalyze the production of nitric oxide (NO) from L-arginine. Nitric oxide (NO) plays an important role in neurotransmission, vascular regulation, immune response and apoptosis. In presence of NADPH, FAD, FMN, (6R)-5,6,7,8-tetrahydrobiopterin, calmodulin and heme, NOS catalyzes a five-electron oxidation of the guanidino nitrogen of L-arginine with molecular oxygen to generate NO and L-citrulline.

There are three isoforms of NOS: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). nNOS accounts for the production of NO in central nervous system, where NO participates in cell communication and information storage. eNOS produces NO in blood vessels and is involved in regulation of vascular function. In contrast to other isoforms, iNOS is expressed *de novo* under oxidative stress conditions and produces large amounts of NO as a part of body's defense mechanism.

2. Protocol Summary



3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipette by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
NOS Assay Buffer	100 mL	-20°C	4°C/-20°C
Staining Dye (in DMSO)	20 µL	-20°C	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Fluorescence microplate reader capable of measuring fluorescence at Ex/Em = 485/530 nm
- Standard fluorescence microscope equipped with a filter set compatible with fluorescein (Ex/Em = 485/530 nm)
- General tissue culture supplies
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- For microplate assay: 96 well plate with clear flat bottom, preferably black
- For fluorescence microscopy: 96 well plate with clear flat bottom

8. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 NOS assay buffer (100 mL):

Ready to use as supplied. Warm to 37°C before use. Store at 4°C or -20°C.

9.2 Staining Dye (in DMSO, 20 µL):

Ready to use as supplied. Warm by placing in a 37°C bath for 1-5 minutes to thaw DMSO solution before use.

Δ Note: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for a few minutes at 37°C.

Aliquot dye so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light.

10. Assay Procedure – Microplate format

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.
- Each cell line should be evaluated on an individual basis to determine the optimal cell density.

10.1 Grow and treat cells:

10.1.1 In a 96 well plate, culture 5-10 x 10^4 cells /well in 200 µL of desired media.

10.1.2 Treat the cells with compound(s) of interest in 200 µL media.

Δ Note: As control, we recommend treating cells with vehicle alone.

10.1.3 Incubate cells overnight in a 37°C incubator (5% CO_2).

10.2 Washing step – adherent cells:

10.2.1 Carefully remove the media without disturbing the cells.

10.2.2 Gently wash the cells twice with 200 µL Assay Buffer each.

10.2.3 Proceed to step 10.4.

10.3 Washing step – suspension cells:

10.3.1 Centrifuge the plate at 1000 x *g* for 5 minutes.

10.3.2 In the absence of a plate centrifuge, carefully transfer the cells in a 1.5 mL conical tube and centrifuge the tube at 1000 x *g* for 5 minutes.

10.3.3 Carefully remove the media without disturbing the cells.

10.3.4 Gently resuspend the cells in 200 µL Assay Buffer.

10.3.5 Repeat the wash step.

10.3.6 Proceed to step 10.4.

Δ Note: The wash steps are necessary to remove serum, BSA, or phenol red from the samples as these components affect the fluorescent signal.

10.4 Staining step:

10.4.1 Prepare a 1X Working Solution of the Staining Dye immediately prior use by diluting dye 1:200 with the Assay Buffer.

10.4.2 Add 20 µL/well of diluted Staining Dye to each well containing cells.

10.4.3 Incubate plate for 1 h at 37°C in the incubator, protected from light.

Δ Note: Since the Staining Dye photo-bleaches very rapidly, we recommend analyzing cells immediately.

10.5 Analyze cells:

10.5.1 Measure fluorescence immediately on a plate reader at Ex/Em =485/530 nm.

11. Assay Procedure – Fluorescence microscopy

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.
- Each cell line should be evaluated on an individual basis to determine the optimal cell density.

11.1 Grow and treat cells:

11.1.1 In a 96 well plate, culture 5-10 x 10^4 cells /well in 200 µL of desired media.

11.1.2 Treat the cells with compound(s) of interest in 200 µL media.

Δ Note: As control, we recommend treating cells with vehicle alone.

11.1.3 Incubate cells overnight in a 37°C incubator (5% CO_2).

11.2 Washing step – adherent cells:

11.2.1 Carefully remove the media without disturbing the cells.

11.2.2 Gently wash the cells twice with 200 µL Assay Buffer each.

11.2.3 Proceed to step 10.4.

11.3 Washing step – suspension cells:

11.3.1 Centrifuge the plate at 1000 x *g* for 5 minutes.

11.3.2 In the absence of a plate centrifuge, carefully transfer the cells in a 1.5 mL conical tube and centrifuge the tube at 1000 x *g* for 5 minutes.

11.3.3 Carefully remove the media without disturbing the cells.

11.3.4 Gently resuspend the cells in 200 µL Assay Buffer.

11.3.5 Repeat the wash step.

11.3.6 Proceed to step 10.4.

Δ Note: The wash steps are necessary to remove serum, BSA, or phenol red from the samples as these components affect the fluorescent signal.

11.4 Staining step:

11.4.1 Prepare a 1X Working Solution of the Staining Dye immediately prior use by diluting dye 1:200 with the Assay Buffer.

11.4.2 Add 20 µL/well of diluted Staining Dye to each well containing cells.

11.4.3 Incubate for 1 h at 37°C in the incubator, protected from light).

Δ Note: Do not stored stained cells as the Staining Dye photo-bleaches very rapidly. We recommend analyzing cells immediately.

11.5 Analyze cells:

11.5.1 Wash the cells carefully to remove excess dye and add 200 µL assay buffer/well.

11.5.2 Examine cells immediately using a light/fluorescence microscope equipped with a filter for Ex/Em = 485/530 nm.

12. Data Analysis

FLUORESCENCE MICROPLATE MEASUREMENT

- Subtract plate blank readings from all measurements (control and treated).
- Using fluorescent intensity, determine fold change between control and treated cells.

FLUORESCENCE MICROSCOPY MEASUREMENT

- We recommend acquiring several images per well.
- We recommend data analysis after coding and mixing images to ensure unbiased results.
- For manual analysis, if you do not have a specific software installed in your microscope, you can download ImageJ, an open source image processing designed for scientific multidimensional images by the National Institute of Health (NIH).

13. Typical Data

Typical detection data provided for **demonstration purposes** only.

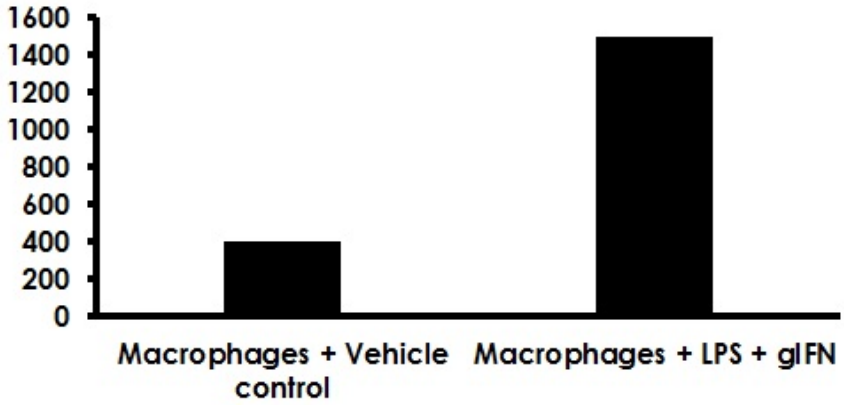


Figure 1. Nitric Oxide Synthase (NOS) detection in J744.1A macrophages using a microplate reader. Macrophages were cultured overnight and treated the next day with either vehicle control (no stimulation) or LPS (200 ng/mL) and IFN-gamma (100 ng/mL) for 24 hours. After washing with Assay Buffer, cells were stained with the Staining Dye for 1 hour at 37°C. The fluorescence signal was measured at Ex/Em = 485/530 nm.

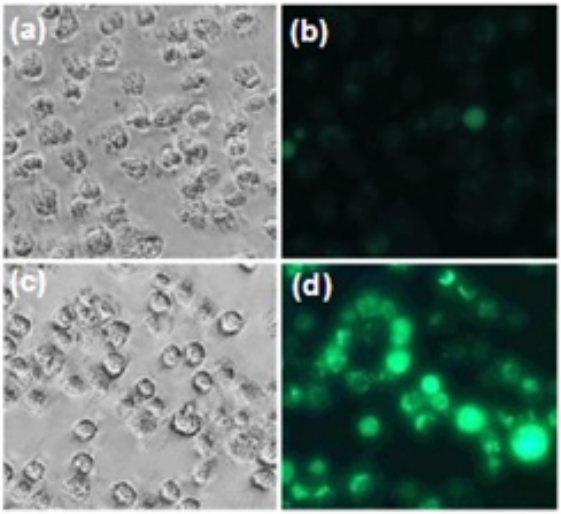


Figure 2. NOS detection in J744.1A macrophages using a fluorescence microscope. Macrophages were cultured overnight and treated the next day with either vehicle control (no stimulation) or LPS (200 ng/mL) and IFN-gamma (100 ng/mL) for 24 hours. After washing with Assay Buffer, cells were stained with the Staining Dye for 1 hour at 37°C. Cells were imaged using a Nikon TiE microscope. Control cells (vehicle treated) are shown in the upper panel [(a), (b)]. Treated cells are shown in the lower panel [(c), (d)].

14. Troubleshooting

Problem	Reason	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at assay temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different microplate	Colorimetric: clear plates Fluorometric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates
Sample with erratic readings	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ dilute sample so it is within the linear range

15. Interferences

These chemical or biological materials will cause interferences in this assay causing compromised results or complete failure:

- Serum, BSA, or phenol red present in the cell culture media will affect the fluorescent signal. Wash cells in Assay Buffer.

16. Notes

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