Version 7, Last updated 18 March 2024

ab211086 Nitric Oxide Synthase Inhibitor Assay Kit (Fluorometric)

For the rapid, sensitive and accurate screening of potential Nitric Oxide Synthase (NOS) inhibitors.

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

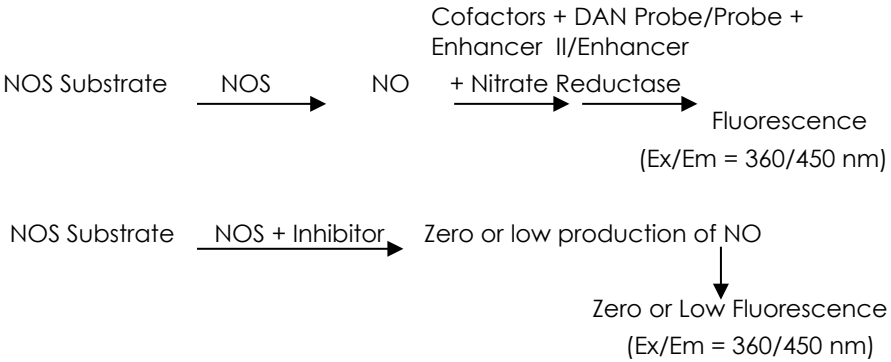
PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

Table of Contents

1. Overview

Nitric Oxide Synthase Inhibitor Assay Kit (Fluorometric) (ab211086) provides a rapid, simple, sensitive and reliable test suitable for high-throughput screening of nitric oxide synthase (NOS) inhibitors. In this assay, nitric oxide (NO) generated by NOS undergoes a series of reactions and reacts with the fluorescent probe to generate a stable signal at Ex/Em = 360/450 nm, which is directly proportional to NOS activity. In the presence of a NOS-specific inhibitor, the formation of NO is reduced/abolished resulting in decrease or total loss of the fluorescence.

This simple and high-throughput adaptable assay can be used to screen/study/characterize potential inhibitors of NOS.

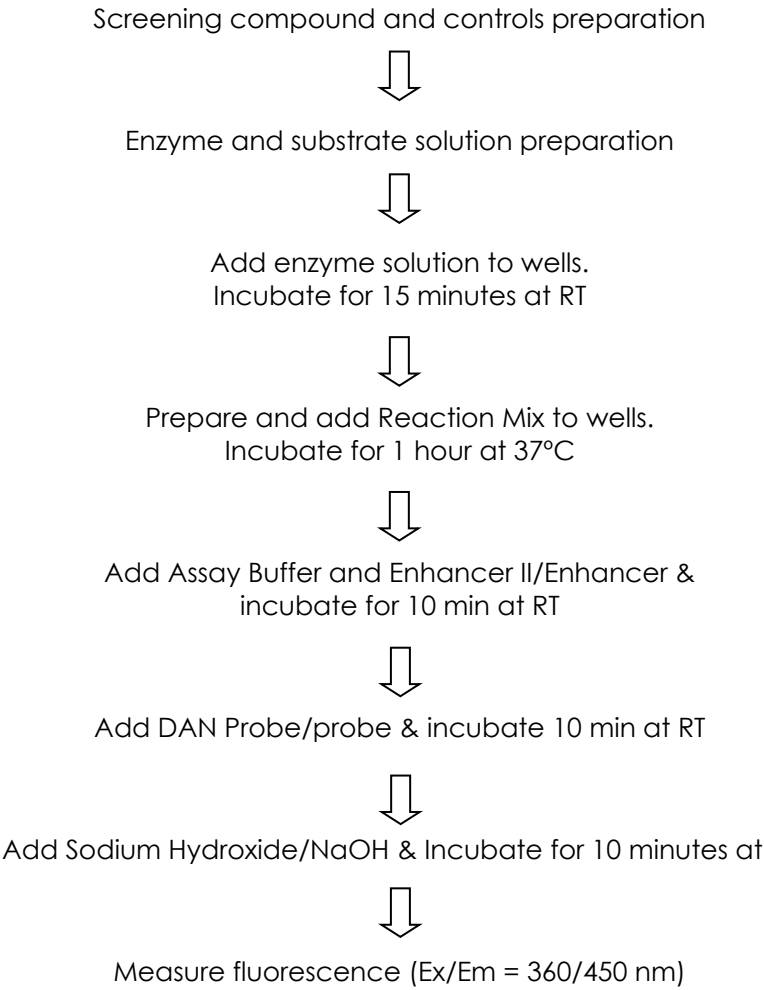


Nitric oxide synthases (EC 1.14.13.39) (NOS) are a family of enzymes that catalyze the production of nitric oxide (NO) from L-arginine. Nitric oxide (NO) plays an important role in neurotransmission, vascular regulation and apoptosis. In presence of NADPH, FAD, FMN, (6R)-5,6,7,8-tetrahydrobiopterin, 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 -80°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	25 mL	-80°C	4°C/ -20°C
NOS Dilution Buffer	1.5 mL	-80°C	4°C/ -20°C
NOS Enzyme	1 vial	-80°C	-80°C
NO Inhibitor (DPI)/NOS Inhibitor (DPI chloride1 mM)	20 µL	-80°C	-20°C
NOS Substrate	500 µL	-80°C	-20°C
Nos Cofactor 1/NOS Cofactor 1 (Lyophilized)	1 vial	-80°C	-20°C
25X NOS Cofactor 2/NOS Cofactor 2	100 µL	-80°C	-20°C
Nitrate Reductase II/Nitrate Reductase (Lyophilized, 1U)	1 vial	-80°C	-20°C
Enhancer II/Enhancer (Lyophilized, 0.5 µmole)	1 vial	-80°C	-20°C
DAN Probe/Probe	1 mL	-20°C	4°C/ -20°C
Sodium Hydroxide/NaOH	1 mL	-80°C	4°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:

- Microplate reader capable of measuring fluorescence at Ex/Em = 360/450 nm
- Deionized water or other type of double distilled water (ddH_2O)
- 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
- 96 well plate with black wells and 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, controls 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 (25 mL):

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C or -20°C.

9.2 NOS Dilution Buffer (1.5 mL):

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C or -20°C.

9.3 NOS Enzyme:

Keep the solution on ice at all times while in use since the enzyme loses activity at higher temperature. Immediately prior use, dilute NOS Enzyme by adding 400 µL NOS Dilution Buffer to the vial. Divide the NOS Enzyme working solution into aliquots and store at -80°C. Limit freeze/thaw to 1 time.

9.4 NO Inhibitor (DPI)/NOS Inhibitor (DPI, diphenyleneiodonium chloride, 1 mM) (20 µL):

Dilute to 1:5 with NOS Assay Buffer just before use. Keep on ice while in use Aliquot inhibitor so that you have enough volume to perform the desired number of assays. Store at -20°C.

9.5 NOS Substrate (500 µL):

Ready to use as supplied. Keep on ice while in use. Aliquot substrate so that you have enough to perform the desired number of assays. Avoid repeated freeze/thaw. Store at -20°C.

9.6 Nos Cofactor 1/NOS Cofactor 1 (lyophilized):

Reconstitute NOS Cofactor 1 in 110 µL of ddH_2O to make a 10 mM stock solution. Aliquot so that you have enough to perform the desired number of assays. Store at -20°C. Limit freeze/thaw to 1 time.

Just before use, make dilute the10 mM stock 1:6 in ddH_2O to make a **1.66 mM Working Solution**. Make as much working solution as needed. Keep on ice while in use. Working solution can be stored at 4°C for 6 – 8 hours.

9.7 25X NOS Cofactor 2/NOS Cofactor 2 (100 µL):

Just before use, dilute Nos Cofactor 1/NOS Cofactor 1:100 in ddH_2O. Make as much as needed for the assay. Keep on ice while in use.

Aliquot undiluted solution so that you have enough volume to perform the desired number of assays. Store at - 20°C. Avoid repeated freeze/thaw.

9.8 Nitrate Reductase II/Nitrate Reductase (Lyophilized, 1U):
Reconstitute Nitrate Reductase II/Nitrate Reductase in 1.1 mL of NOS Assay Buffer. Keep on ice while in use. Store at -20°C. Avoid repeated freeze/thaw.

9.9 Enhancer II/Enhancer (Lyophilized, 0.5 µmole):
Reconstitute Enhancer II/Enhancer in 1.2 mL of NOS Assay Buffer. Aliquot Enhancer II/enhancer so that you have enough to perform the desired number of assays. Keep on ice while in use. Store at -20°C.

9.10 DAN Probe/Probe (1 mL):
Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C or -20°C.

9.11 Sodium Hydroxide/NaOH (1 mL):
Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C or -20°C.

10. Sample Preparation

General sample information:

– Always prepare a fresh set of samples and controls for every use.

10.1 Screening compounds

10.1.1 Dissolve test compounds into proper solvent.
10.1.2 Dilute to 4X the desired test concentration with NOS Assay Buffer before use.

Δ Note: We suggest using different concentrations of test compounds if effective concentration is unknown.

11. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.

Δ Note: preferred final solvent concentration should not be more than 5% by volume. If solvent exceeds 5%, include solvent control to test the effect of the solvent on enzyme activity.

11.1 Set up Reaction wells:

11.1.1 Add 4 µL of NOS Enzyme working solution (Step 9.3) to all wells except background control.

11.1.2 Prepare reaction wells as follows:

- Sample compound wells (S) = 4 µL NOS Enzyme working solution + 10 µL test compounds + 16 µL NOS Assay Buffer.
- Inhibitor Control wells (IC) = 4 µL NOS Enzyme working solution + 10 µL diluted NO Inhibitor (DPI)/NOS Inhibitor provided (DPI) + 16 µL NOS Assay Buffer.
- Enzyme Control wells (EC) = 4 µL NOS Enzyme working solution + 26 µL NOS Assay Buffer.
- Background control (BC) = 30 µL NOS Assay Buffer.
- OPTIONAL: Solvent control (SC) = 4 µL NOS Enzyme working solution + 10 µL of 4X Solvent(s) + 16 µL NOS Assay Buffer.

11.1.3 Incubate at room temperature for 15 minutes.

11.2 NOS Reaction Mix:

11.2.1 Prepare 11 µL of Reaction Mix for each well to be analyzed. Mix enough reagents for the number of assays to be performed. Prepare a master mix to ensure consistency.

Component	NOS Reaction Mix (µL)
Diluted NOS Cofactor 1	3
Diluted NOS Cofactor 2	1
NOS Substrate	2
Nitrate Reductase II/Nitrate	5

Reductase	

11.2.2 Mix and add 11 µl of the Reaction Mix into each well and incubate at 37°C for 1 hour.

11.3 Measurement:

11.3.1 After incubation, add 110 µL of NOS assay buffer into each well and subsequently add 5 µL of the Enhancer II/enhancer into each well.

11.3.2 Mix and incubate at room temperature for 10 minutes.

11.3.3 Add 10 µL of DAN Probe/Probe to all wells. Mix and incubate for 10 minutes.

11.3.4 Add 5 µL of Sodium Hydroxide/NaOH to all wells. Mix and incubate for 10 minutes.

11.3.5 Measure fluorescence immediately on a microplate reader at Ex/Em = 360/450 nm.

12. Calculations

– Use only the linear rate for calculation.

12.1 Plot readings for each sample test compound (S), inhibitor control (IC) and enzyme control (EC).

12.2 Draw the line of the best fit to construct the curve (most plate reader software or Excel can do this step). Calculate the trend line equation (use the equation that provides the most accurate fit).

12.3 Average the duplicate reading for each sample test compound (S), inhibitor control (IC) and enzyme control (EC).

12.4 Subtract Background Control (BC) reading from the Enzyme Control (EC) and Inhibitor (S). If the data obtained from the solvent control(s) is significantly different from the EC use this data instead of EC data in the equation below.

$$\%\ Relative\ Inhibition = \frac{RFU(EC) - RFU(S)}{RFU(EC)}\ x\ 100$$

Δ Note: Irreversible inhibitors that inhibit NOS activity completely at the tested concentration will have RFU = 0 and thus % Relative Inhibition will be 100%.

Δ Note: If RFU of SC < RFU of EC = make a higher stock of test inhibitor or dissolve the inhibitor in lower concentration of the solvent; or use a different solvent if possible.

If RFU of S < RFU of EC = treat as 100% inhibition and further dilute the test inhibitor and repeat the assay.

13. Typical Data

Data provided **for demonstration purposes only.**

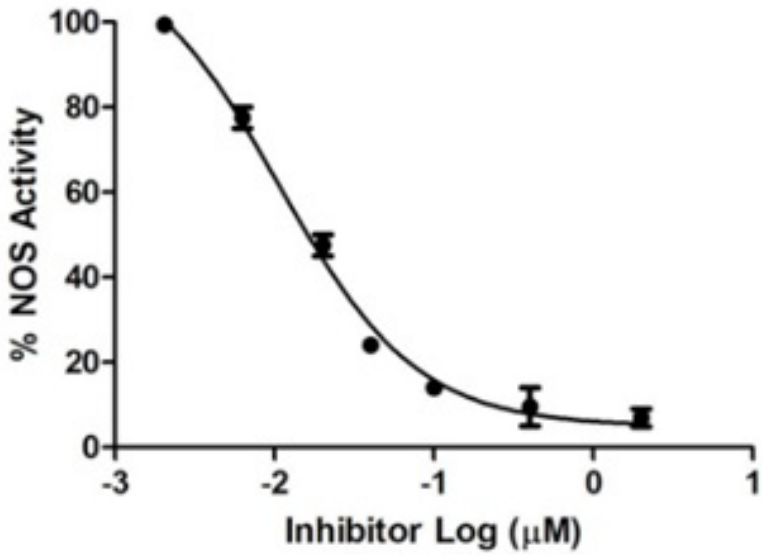


Figure 1. Inhibition of NOS Enzyme activity by diphenyleneiodonium chloride (DPI). IC_{50} of Diphenyleneiodonium chloride was determined to be 20 nM. Assay was performed following the kit protocol.

14. Quick Assay Procedure

Δ Note: this procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare assay components; get equipment ready.
- Prepare and dilute test compounds in suitable solvent.
- Prepare NOS substrate Reagent Mix (11 µL/well) for all wells:

Component	Reaction Mix (µL)
Diluted NOS Cofactor 1	3
Diluted NOS Cofactor 2	1
NOS Substrate	2
Nitrate Reductase II/Nitrate Reductase	5

- Set up plate as follows:

Component	Sample (S) (µL)	Solvent control (SC) (µL)	Enzyme Control (EC) (µL)	Inhibitor Control (IC) (µL)	Bckg Control (BC) (µL)
NOS Enzyme working solution	4	4	4	4	0
Test compound	10	0	0	0	0
NO Inhibitor/NOS inhibitor control	0	0	0	10	0
Solvent compound	0	10	0	0	0
Assay Buffer	16	16	26	16	30
Incubate 15 min RT					
Add 11 µL NOS reaction mix and incubate 1 h 37°C					
Add 110 µL NOS assay buffer					
Add 5 µL Enhancer II/enhancer and incubate 10 min RT					
Add 10 µL DAN Probe/Probe and incubate 10 min RT					
Add 5 µL Sodium Hydroxide/NaOH and incubate 10 RT					

- Measure fluorescence at Ex/Em = 360/450 nm.

15. 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
Assay with erratic readings	Pipetting errors	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
No fluorescence above background in inhibitor wells	Inhibitor concentration is too high	Reduce concentration of inhibitor and re-do assay
No inhibition seen in test compound wells	Inhibitor concentration is not high enough	Increase concentration of inhibitor and re-do assay
	Compound is not an inhibitor	Use another compound for your test

16. Notes

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)