

Version 5 Last updated 27 October 2023

ab211083

Nitric Oxide Synthase Activity Assay Kit (Colorimetric)

For the rapid, sensitive and accurate measurement of Nitric Oxide Synthase (NOS) activity in cell and tissue extracts.

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.

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1. Overview

Nitric Oxide Synthase (NOS) Activity Assay Kit (Colorimetric) (ab211083) provides an accurate and convenient method to assay NOS activity in cell and tissue lysates. In this assay, nitric oxide generated by NOS undergoes a series of reactions and reacts with Griess Reagent I /Griess Reagent 1 and 2 to generate a colored product with a strong absorbance at OD 540 nm.

The assay is simple, sensitive and high-throughput adaptable and can detect as low as 5 μ U of NOS activity.

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

Standard curve preparation



Sample preparation



Add reaction mix &
incubate for 1h at 37°C



Add Assay Buffer and Enhancer II/Enhancer &
incubate for 10 min at RT



Add Griess Reagent I /Griess Reagent 1 and Griess Reagent
II/Reagent 2



Incubate for 10 minutes at RT



Measure optimal density (OD540 nm)

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.

Δ Note: Reconstituted components are stable for 2 months.

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)	reaction 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 Substrate	500 µL	-80°C	-20°C
NOS Cofactor I/NOS Cofactor 1 (1 µmole)	1 vial	-80°C	-20°C
25X NOS Cofactor 2/NOS Cofactor 2 (25X)	100 µL	-80°C	-20°C
Nitrate Reductase II/Nitrate Reductase (1 U)	1 vial	-80°C	-20°C
NOS Positive Control/NOS (Positive Control)	4 µL	-80°C	-80°C
Enhancer II/Enhancer (0.5 µmole)	1 vial	-80°C	-20°C
Nitrite Standard/Nitrite Standard (10 µmole)	1 vial	-80°C	4°C
Griess Reagent I /Griess Reagent 1	10 mL	-80°C	4°C
Griess Reagent II/Griess Reagent 2	10 mL	-80°C	4°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 absorbance at OD 540 nm
- Deionized water or other type of double distilled water (ddH₂O)
- PBS
- 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 clear flat bottom
- Dounce homogenizer (if using tissue)
- BCA protein assay kit (reducing agent compatible): we recommend using BCA protein assay kit reducing agent compatible (microplate) (ab207003)
- Protease inhibitor cocktail: we recommend Protease Inhibitor Cocktail (ab65621)

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, standard 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.
- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- 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 Substrate (500 µL):

Ready to use as supplied. Keep on ice while in use. Divide into aliquot substrate so that you have enough to perform the desired number of assays. Store at -20°C.

9.4 NOS Cofactor I/NOS Cofactor 1 (lyophilized, 1 µmole):

Reconstitute NOS Cofactor 1 in 110 µL of ddH₂O to make 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 **1mM Working Solution NOS Cofactor 1** by diluting the 10 mM stock in dH₂O. 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.

9.5 25X NOS Cofactor 2/NOS Cofactor 2 (25X) (100 µL):

Just before use, make **1X Working Solution NOS Cofactor 2** by diluting stock in ddH₂O. Make as much working solution as needed. Keep on ice while in use.

Divide stock solution(25X) into aliquots so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze/thaw cycles.

9.6 Nitrate Reductase II/Nitrate Reductase (lyophilized, 1 U):

Reconstitute Nitrate Reductase II/Nitrate Reductase in 1.1 mL of NOS Assay Buffer. Keep on ice while in use. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze/thaw.

9.7 NOS Positive Control/NOS (Positive Control) (4 µL):

Keep the solution on ice at all times while in use since the enzyme loses activity at higher temperature. Immediately prior use, dilute NOS Positive Control/NOS 1:20 in NOS Dilution Buffer. Aliquot enzyme so that you have enough to perform the desired number of assays. Store at -80°C. Limit freeze/thaw to 1 time.

9.8 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.

9.9 Nitrite Standard/Nitrite Standard (lyophilized, 10 µmole):

Reconstitute Nitrite Standard in 1 mL of NOS Assay Buffer to generate a 10 mM standard. Vortex and mix well. Do not freeze. Store at 4°C. Reconstituted Standard is stable for 4 months when stored at 4°C.

9.10 Griess Reagent I /Griess Reagent 1 (10 mL):

Ready to use. Store at 4°C.

9.11 Griess Reagent II/Griess Reagent 2 (10 mL):

Ready to use. Store at 4°C.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

10.1 Prepare a 50 μM Nitrite working standard solution by adding 5 μL of 10mM Nitrite Standard to 995 μL of NOS Assay Buffer. Mix well.

10.2 Using 50 μM Nitrite working standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Nitrite Standard (μL)	Assay Buffer (μL)	Final volume standard in well (μL)	End amount Nitrite in well (pmol/well)
1	0	180	60	0
2	15	165	60	250
3	30	150	60	500
4	45	135	60	750
5	60	120	60	1000
6	75	105	60	1250

Each dilution has enough amount of standard to set up duplicate readings (2 x 60 μL).

11. Sample Preparation

General sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C . When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
- Add protease inhibitor cocktail to the assay buffer immediately prior to use.

11.1 Cell lysates:

- 11.1.1 Culture the required amount of cells necessary for each assay (initial recommendation $2-5 \times 10^6$ cells).
- 11.1.2 Wash cells with cold PBS.
- 11.1.3 Resuspend cells in $100 - 200 \mu\text{L}$ of cold NOS Assay Buffer (containing protease inhibitor cocktail) on ice. If cells do not detach, a cell scraper may be used.
- 11.1.4 Homogenize cells quickly by pipetting up and down a few times.
- 11.1.5 Centrifuge sample for 10 minutes at 4°C at $10,000 \times g$ using a cold microcentrifuge to remove any insoluble material.
- 11.1.6 Collect supernatant and transfer to a new pre-chilled tube.
- 11.1.7 Keep on ice.
- 11.1.8 Measure the amount of protein in the cell lysate using a BCA Protein Assay kit.
- 11.1.9 Proceed to assay NOS activity immediately.

11.2 Tissue lysates:

- 11.2.1 Harvest the amount of fresh or frozen tissue necessary for each assay (initial recommendation $\sim 100 \text{ mg}$).
- 11.2.2 Wash tissue in cold PBS.
- 11.2.3 Add $200 \mu\text{L}$ of cold NOS Assay Buffer (containing protease inhibitor cocktail) to tissue.

- 11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.
- 11.2.5 Centrifuge sample for 10 minutes at 4°C at 10,000 x *g* using a cold microcentrifuge to remove any insoluble material.
- 11.2.6 Collect supernatant and transfer to a new pre-chilled tube.
- 11.2.7 Keep on ice.
- 11.2.8 Measure the amount of protein in the lysate or purified enzyme using a BCA Protein Assay kit.
- 11.2.9 Proceed to assay NOS activity immediately.

Δ Note: We suggest using different volumes of sample to ensure readings are within the standard curve range.

12. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

Δ Note: If you suspect your samples contain substances that can generate background, set up Sample Background Controls to correct for background noise.

12.1 Plate Loading:

- Standard wells = 60 μ L standard dilutions.
- Positive control = 2- 50 μ L diluted positive control (1:20) (adjust volume to 60 μ L/well with NOS Assay Buffer).
- Sample wells = 30 - 60 μ L (200 – 400 μ g protein) sample into each well (adjust volume to 60 μ L/well with NOS Assay Buffer).
- Sample Background Control wells = 30 - 60 μ L (200 – 400 μ g protein) sample into each well. Adjust final volume to 300 μ L/well with NOS Assay Buffer.

12.2 NOS Reaction Mix:

12.2.1 Prepare 40 μ L of Reaction Mix for each well. Mix enough reagents for the number of assays to be performed. Prepare a master mix to ensure consistency.

Component	Reaction Mix (μ L)
Diluted NOS Cofactor 1	10
NOS Cofactor 2 (1X)	20
NOS Substrate	5
Nitrate Reductase II/Nitrate Reductase	5

- 12.2.2 Add 40 μL of Reaction Mix into standard, positive control and sample well. DO NOT add reaction mix to background control wells.
- 12.2.3 Mix well by pipetting up and down.
- 12.2.4 Incubate at 37°C for 1 hour.
- 12.2.5 Add 95 μL of NOS Assay Buffer to standard, positive control and sample well. DO NOT add reaction mix to background control wells. Mix well by pipetting up and down.
- 12.2.6 Add 5 μL of Enhancer II/Enhancer to standard, positive control and sample well. DO NOT add Enhancer II/enhancer to background control wells.
- 12.2.7 Mix well by pipetting up and down.
- 12.2.8 Incubate at room temperature for 10 minutes.

12.3 Measurement:

- 12.3.1 Add 50 μL of Griess Reagent I /Griess Reagent 1 and 50 μL of Griess Reagent II/Griess Reagent 2 to standard, positive control and sample well. DO NOT add reagents 1 and 2 to background control well.
- 12.3.2 Mix well by pipetting up and down.
- 12.3.3 Incubate at room temperature for 10 minutes.
- 12.3.4 Measure absorbance immediately (for all wells) on a microplate reader at OD 540 nm.

13. Calculations

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.
- Use only the linear rate for calculation.

13.1 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.2 Standard curve calculation:

13.2.1 Average the duplicate reading for each standard.

13.2.2 Plot standard curve readings and draw the line of the best fit to construct the standard curve (most plate reader software or Excel can do this step). Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

13.3 Measurement of NOS in the sample:

13.3.1 Subtract the sample background control from sample reading if significant.

13.3.2 Apply variation of absorbance in the sample readings ($\Delta OD_{540\text{ nm}}$) to the Standard curve to get B pmoles of nitrite generated during the reaction.

13.3.3 Nitrite activity (pmol/min/ μg or mU/mg) in the test samples is calculated as:

$$\text{NOS Specific Activity} = \left(\frac{B}{T \times C} \right)$$

Where:

B = amount of Nitrite amount in sample well calculated from Standard Curve (pmol).

T = Reaction time (minutes) – 60 minutes in this case.

C = is amount of protein (μg)

Unit definition:

1 Unit NOS activity is the amount of enzyme required to yield 1 μmol of nitric oxide/minute at 37°C under the assay conditions.

14. Typical Data

Typical standard curve – data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

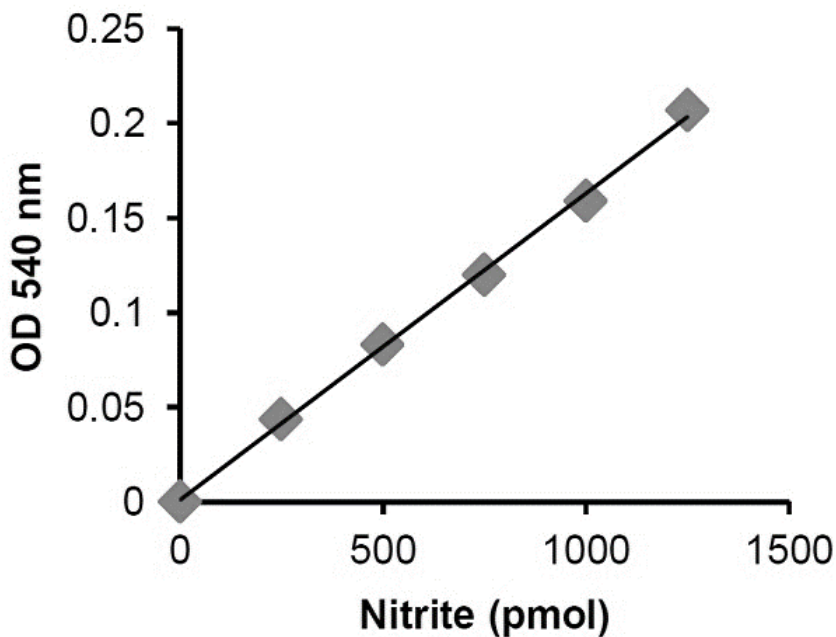


Figure 1. Typical standard calibration curve.

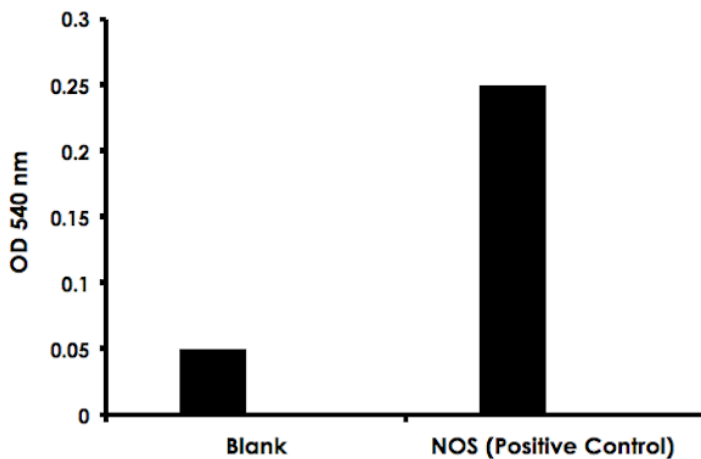


Figure 2. Measurement of NOS Positive Control activity (10 μ l) compared to blank (Standard #1).

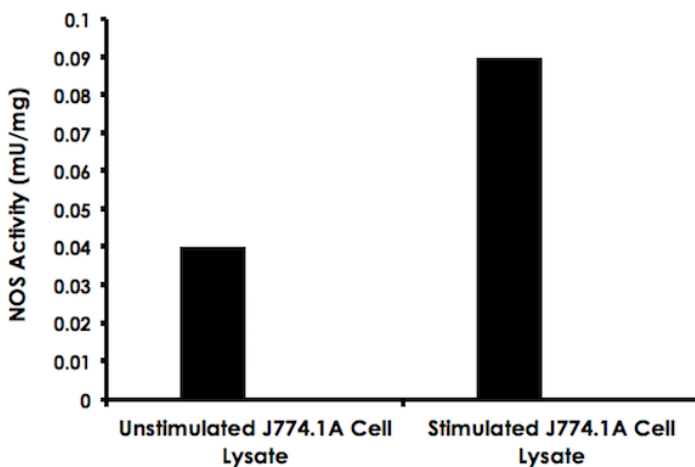


Figure 3. Detection of endogenous NOS activity in J774.1A cell lysate (225 μ g) unstimulated or stimulated with or 200 ng/mL LPS and 100 ng/mL murine IFN- γ .

15. 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 reagents and aliquot if necessary; get equipment ready.
- Prepare NOS standard dilution [250 – 1250 pmol/well].
- Prepare samples in optimal dilutions to fit standard curve readings.
- Set up plate in duplicate for standard (60 μ L), sample (30 - 60 μ L) and positive control wells (60 μ L).

Sample background control wells, use 30 – 60 μ L sample and adjust to 300 μ L with NOS Assay Buffer. Do not add any other reagent to these wells.

- Prepare a master mix for NOS Reaction Mix:

Component	Reaction Mix (μ L)
Diluted NOS Cofactor 1	10
NOS Cofactor 2 (1X)	20
NOS Substrate	5
Nitrate Reductase II/Nitrate Reductase	5

- Add 40 μ L Reaction mix to sample, standard and positive control wells.
- Incubate at 37°C for 60 minutes.
- Add 95 μ L NOS Assay Buffer and 5 μ L Enhancer II/Enhancer to sample, standard and positive control wells.
- Incubate at room temperature for 10 minutes.
- Add 50 μ L Griess Reagent I /Griess Reagent 1 and 50 μ L Griess Reagent II/Griess Reagent 2 to sample, standard and positive control wells.
- Incubate at room temperature for 10 minutes.
- Measure absorbance immediately at OD 540 nm on a microplate reader.

16. 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	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	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
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Reason	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 μ L) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions described in the protocol
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

17. Notes

Technical Support

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