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Product Information

Nitric Oxide Synthase Inhibitor Screening Kit

Catalog Number **MAK323**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Nitric oxide (NO) is a reactive radical that plays an important role in many key physiological functions. NO is the oxidation product of arginine by nitric oxide synthase (NOS) and is involved in host defense development, activation of regulatory proteins, and direct covalent interaction with functional biomolecules. Inhibition of NOS has the potential to produce diverse biological effects, particularly in the cardiovascular system. Simple, direct, and non-radioactive procedures for measuring NOS are becoming popular in research and drug discovery.

The Nitric Oxide Synthase Inhibitor Screening Kit involves two steps: a NOS reaction step during which NO is produced followed by an NO detection step. Since the NO generated by NOS is rapidly oxidized to nitrite and nitrate, the NO production is measured following reduction of nitrate to nitrite using an improved Griess method.

This kit can be readily automated on HTS liquid handling systems and is suitable for inhibitor screening and evaluation of nitric oxide synthase inhibitors.

Components

The kit is sufficient for 100 colorimetric assays in 96 well plates.
Note: Neither the enzyme NOS nor a control inhibitor is included in the kit.

Assay Buffer Catalog Number MAK323A	10 mL
Substrate Catalog Number MAK323B	600 µL
GDH Catalog Number MAK323C	120 µL
Reagent A Catalog Number MAK323D	12 mL
Reagent B Catalog Number MAK323E	500 µL
Reagent C Catalog Number MAK323F	12 mL
Reagent D (Dried) Catalog Number MAK323G	1 Vial
Reagent E Catalog Number MAK323H	1.5 mL

Reagents and Equipment Required but Not Provided.

- Nitric Oxide Synthase, Inducible from mouse (iNOS, Catalog Number N2783)
- If desired, a control inhibitor: Diphenyleneiodonium chloride (DPI, Catalog Number D2926)
- Pipetting devices and accessories (e.g., multichannel pipettor)
- 96 well flat bottom plate. It is recommended to use clear plates for colorimetric assays
- Spectrophotometric multiwell plate reader

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

Preparation Instructions
Use ultrapure water for the preparation of all reagents and samples.

Reagent Preparation
Prior to assay, equilibrate all components to room temperature.

Reagent D Solution: Reconstitute Reagent D with 300 µL of ultrapure water. Store unused Reagent D Solution at –20 °C and use within 1 week.

Assay Buffer: Prewarm to 25 °C.

GDH: Keep GDH on ice prior to use.

Reagent B: Ready to use. If precipitates are present, warm at 37 °C until redissolved (10–15 minutes).

The Reaction Mix should be prepared freshly and used within 30 minutes.

Storage/Stability
The kit is shipped on dry ice. Store Reagents A, B, and C between –20 °C to 4 °C. All other reagents should be stored at –20 °C. Use Reagent D within 1 week after reconstitution.

Procedure
Sample Preparation
NOS Solution - Dilute Nitric Oxide Synthase, Inducible from mouse (iNOS, Catalog Number N2783) to 12.5 U/µL using ultrapure water or diluent.
Note: The following protocol is optimized for iNOS from mouse. If another species is being analyzed, it is recommended that one experimentally determine the K_M value and then adjust the volume of substrate in the Reaction Mix so that the final concentration of the substrate in the 50 µL reaction is near the K_M.

Inhibitor Solution - Dissolve the test compounds (i.e., inhibitors) in solvent of choice. It is prudent to first test the tolerance of the solvent by the enzyme to be inhibited. If using DMSO, the DMSO concentration should be 20% (v/v) or less in the 5 µL of test compounds added to the reaction when screening with iNOS from mouse.

NOS Reaction Preparation
1. Transfer 10 µL of the NOS Solution into separate wells.
2. Reserve at least one NOS Solution well for the Blank (No Substrate), and one for the Control (Without Inhibitor).
3. To the Control and Blank wells, add 5 µL of the solvent used to dissolve the test compounds. For example, if the test compounds are dissolved in 20% (v/v) DMSO, add 5 µL of 20% (v/v) DMSO to these wells.
4. To the remainder of the wells containing NOS, add 5 µL of the test compounds solution.
5. Add 25 µL Assay Buffer to all wells and incubate the plate for 15 minutes at 25 °C.
6. Prepare the Reaction Mixes according to the scheme in Table 1. 10 µL of the appropriate Reaction Mix is required for all wells.

Table 1.
Reaction Mix

Reagent	Sample Volume	Blank Volume
Reagent D Solution	2 µL	2 µL
Reagent E	5 µL	5 µL
Substrate	5 µL	–
Ultrapure water	–	5 µL
GDH	0.5 µL	0.5 µL

7. Add 10 µL of the appropriate Reaction Mix to Sample and Blank wells.
8. Tap plate to mix briefly and thoroughly. Incubate for 60 minutes at 37 °C.
9. Prepare the NO Detection Reagent according to the scheme in Table 2. 200 µL of the NO Detection Reagent is required for all wells.

Table 2.
NO Detection Reagent

Reagent	Volume
Reagent A	100 µL
Reagent B	4 µL
Reagent C	100 µL

10. At the end of the 60 minutes incubation period (see Step 8), immediately add 200 µL of NO Detection Reagent to each well.
11. Incubate the detection reaction at 37 °C for 60 minutes.
12. Measure the absorbance at 540 nm (A_{540}).

Results

NOS inhibition for a test compound is calculated as follows:

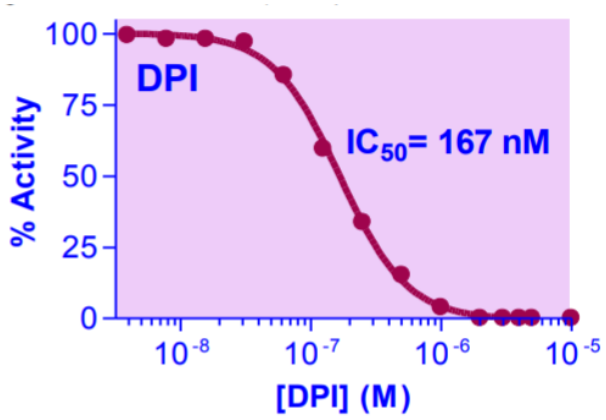
$$\% \text{ Inhibition} = (1 - \Delta A_{\text{Test Cpd}}/\Delta A_{\text{No Inhibitor}}) \times 100\%$$

$\Delta A_{\text{Test Cpd}}$ = the A_{540} value of a test compound minus the A_{540} value of the Blank well (No Substrate) at 60 minutes

$\Delta A_{\text{No Inhibitor}}$ = the A_{540} value of the Control well (No Inhibitor) minus the A_{540} value of the Blank well (No Substrate) at 60 minutes

Figure 1.

DPI inhibition of iNOS from mouse



iNOS from mouse was incubated with various concentrations of DPI. Each concentration of inhibitor contained 20% (v/v) DMSO (final 2% (v/v) in 50 µL reaction).

References

1. Víteček, Jan, et al., Arginine-based inhibitors of nitric oxide synthase: therapeutic potential and challenges. Mediators of Inflammation, (2012), Article ID 318087.
2. Mendes, A.F. et al., Diphenyleneiodonium inhibits NF-κB activation and iNOS expression induced by IL-1β: involvement of reactive oxygen species. Mediators of Inflammation, **10(4)**, 209-215 (2001).
3. Boer, R. et al., The inhibitory potency and selectivity of arginine substrate site nitric-oxide synthase inhibitors is solely determined by their affinity toward the different isoenzymes. Molecular Pharmacology, **58(5)**, 1026-1034 (2000).

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