Nitric Oxide Synthase Assay Kit

Nitric Oxide Assay Kit is a detection kit for the quantification of Nitric Oxide Synthase activity in serum, plasma, whole blood, cell culture media, tissue lysates and cell lysates.

Catalog number: ARG81188

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INTRODUCTION

Nitric oxide synthases (EC 1.14.13.39) (NOSs) are a family of enzymes catalyzing the production of nitric oxide (NO) from L-arginine. NO is an important cellular signaling molecule. It helps modulate vascular tone, insulin secretion, airway tone, and peristalsis, and is involved in angiogenesis and neural development. It may function as a retrograde neurotransmitter. Nitric oxide is mediated in mammals by the calcium-calmodulin controlled isoenzymes eNOS (endothelial NOS) and nNOS (neuronal NOS). The inducible isoform, iNOS, is involved in immune response, binds calmodulin at physiologically relevant concentrations, and produces NO as an immune defense mechanism, as NO is a free radical with an unpaired electron. It is the proximate cause of septic shock and may function in autoimmune disease.. [Wikipedia]

PRINCIPLE OF THE ASSAY

This assay kit is based on a simple, direct and non-radioactive procedures for measuring NOS by two steps reactions: 1) a NOS reaction step 2) 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. The procedure is simple and the time required for sample pretreatment and assay is reduced to as short as 40 min. The intensity of the color is measured at a wavelength of 540 nm. The activity of NOS in the sample is then calculated with the O.D. of samples.

MATERIALS PROVIDED & STORAGE INFORMATION

Component	Quantity	Storage information
Assay Buffer	12 ml	-20°C
Substrate	600 µl	-20°C
GDH	120 µl	-20°C
ROS reaction buffer 1	2 vials (lyophilized)	-20°C
ROS reaction buffer 2	1.5 ml	-20°C
NO Measurement buffer A	12 ml	4°C or -20°C
NO Measurement buffer B	500 µl	4°C or -20°C
NO Measurement buffer C	12 ml	4°C or -20°C
Standard (1.0 mM)	1 ml	-20°C
$ZnSO_4$	1 ml	4°C or -20°C
NaOH	1 ml	4°C or -20°C

The kit is shipped with blue ice. Store the Assay Buffer, Substrate, GDH, ROS reaction buffer 1, ROS reaction buffer 2 and Standard at -20°C and all other reagents at 4°C or -20°C. Shelf life of six months after receipt. Use OS reaction buffer 1 within 1 week after reconstitution.

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 540 nm
- Flat bottomed 96-well microplate
- Eppendorf tubes
- Pipettes and pipette tips
- Heat block or hot water bath or oven
- 1 X PBS (pH 7.4)
- Deionized or distilled water

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C or -20°C at all times. Store the Assay Buffer, Substrate, GDH, ROS reaction buffer 1, ROS reaction buffer 2 and Standard at -20°C only.
- Briefly spin down the reagents before use.
- All reagents should be equilibrated to room temperature (RT, 21°C to 26°C) before assay.
- If crystals or precipitates are observed in the NO Measurement buffer B, warm at 37°C for 10-15 min or until the crystals or precipitates are completely dissolved.
- It is highly recommended that the standards and samples be assayed in at least duplicates.
- Change pipette tips between the addition of different reagent or samples.
- All reagents should be warmed to room temperature before use.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g at 2-8°C. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid

repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g at 2-8°C within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Culture Supernatants - Remove particulates by centrifugation (1000 X g, 10 min) at 2-8°C and aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Tissue or cell samples- Samples are homogenized in 1 X PBS (pH 7.4). Centrifuge at 14,000 rpm at 4°C. Use supernatant for following NOS assay.

Note: Antioxidants and nucleophiles (e.g. β-mercaptoethanol, glutathione, dithiothreitol and cysteine) may interfere with this assay. Avoid using these compounds during sample preparation. However, If β-mercaptoethanol or dithiothreitol must be used, an equal concentration needs to be added to the standards.

REAGENT PREPARATION

- Samples:
  - Samples from serum, plasma, whole blood, cell culture media, tissue lysate and cell lysates need additional deproteination process, and the used standards in the assay also need to through deproteination process.
  - Samples from purified NOS do not need the additional deproteination process.

- **Standards**: Add 100 µl of 1000 µM stock standard into 100 µl distilled water to generate a standard S1 with 500 µM of standard. Dilute the standards with distilled water and distilled water serves as zero standard (blank standard, 0 µM).

  The example of the standards dilution table is as below:

No	Standard (µM)	Volume of Distilled water (µl)	Volume of 500 µM standard (µM)
S1	500	0	50
S2	300	20	30
S3	150	35	15
S4	0	50	0

- **ROS reaction buffer 1:** Reconstitute each vial of ROS reaction buffer 1 with 300 µl of distilled water. If assaying more than 60 wells, reconstitute both vials of ROS reaction buffer 1. Store unused reconstituted ROS reaction buffer 1 at -20˚C and use within 1 week.
- **Assay Buffer:** Prewarm Assay Buffer to 37°C prior assay.

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT) before use. If crystals or precipitates are observed in the NO Measurement Reagent B, warm at 37°C until completely dissolved (around 10-15 min). It is recommended that Standards and samples should be assayed in at least duplicates.

A. For samples requiring deproteination (serum, plasma, whole blood, cell culture media, tissue lysate and cell lysates)

NOS Reaction:

1. Add 25 µL of each sample and standard to separate labeled eppendorf tubes. Each sample requires at least two tubes: one for reaction tube (SR) and another one for sample blank (SB) tube.
2. Immediately prior to starting the reaction, prepare enough NOS working reagent (NOS WR) for all sample reaction tubes (SR) and standards:

   For each reaction tube:

   Assay Buffer: 80 µl

   Substrate: 5 µl

   ROS reaction buffer 1: 5 µl

   ROS reaction buffer 2: 13 µl

   GDH: 1 µl
3. Prepare enough NOS blank reagent (NOS BR) for all sample blank tubes (SB):

   For each blank tube:

   Assay Buffer: 80 µl

dH2O: 10 µl

(Note: instead of Substrate and ROS reaction buffer 1)

ROS reaction buffer 2: 13 µl

GDH: 1 µl

4. Add 100 µL of the appropriate NOS WR or NOS BR to each tube and incubate at 37°C for 20 min. After 20 min immediately proceed to the deproteination step.

Deproteination:

5. Mix 7 µl of $ZnSO_4$ to each sample or standard tube from **NOS reaction step**, vortex to mix complexly.
6. Add 7 µl NaOH in the tube and vortex again.
7. Centrifuge the tubes for 10 minutes at 14,000 rpm in RT.
8. Transfer 100 µl of the clear supernatant to a clean tube for the following assay procedure.

NO Measurement:

1. Add 100 µl of each sample and standard from **Deproteination step** to separate, labeled microcentrifuge tube.
2. Immediately prior to starting the reaction step, prepare enough working reagent for all samples and standards.

   For each reaction tube:

   NO Measurement Reagent A: 100 µl

   NO Measurement Reagent B: 4 µl

   NO Measurement Reagent C: 100 µl
3. Add 200 µl of the prepared NO Measurement working reagent (NO MR) to each sample and standard tube and incubate for 5 min at 60°C.

(Alternatively, incubate at 37°C for 60 min or RT for 150 min.)

4. Briefly centrifuge the reaction tubes to pellet any condensation and transfer 250 µl of each reaction to separate wells in a 96 well plate.
5. Read O.D. with a microplate reader at 540 nm (500-570 nm) immediately.

B. For samples NOT requiring deproteination (purified NOS protein)

NOS Reaction:

1. Add 20 µL of each sample and standard to separate labeled eppendorf tubes. Each sample requires at least two tubes: one for reaction tube (SR) and another one for sample blank (SB) tube.
2. Immediately prior to starting the reaction, prepare enough NOS working reagent (NOS WR) for all sample reaction tubes (SR) and standards:

   For each reaction tube:

   Assay Buffer: 65 µl

   Substrate: 4 µl

   ROS reaction buffer 1: 4 µl

   ROS reaction buffer 2: 10 µl

   GDH: 1 µl
3. Prepare enough NOS blank reagent (NOS BR) for all sample blank tubes (SB):

   For each blank tube:

   Assay Buffer: 65 µl

   dH2O: 8 µl

(Note: instead of Substrate and ROS reaction buffer 1)

ROS reaction buffer 2: 10 µl

GDH: 1 µl

4. Add 80 µL of the appropriate NOS WR or NOS BR to each tube and incubate at 37°C for 20 min. After 20 min immediately proceed to the deproteination step.

NO Measurement:

1. Immediately prior to starting the reaction step, prepare enough working reagent for all samples and standards.

   For each reaction tube:

   NO Measurement Reagent A: 100 µl

   NO Measurement Reagent B: 4 µl

   NO Measurement Reagent C: 100 µl

2. Add 200 µl of the prepared NO Measurement working reagent (NO MR) to each sample and standard tube from NOS Reaction and incubate for 5 min at 60°C. (Alternatively, incubate at 37°C for 60 min or RT for 150 min.)
3. Briefly centrifuge the reaction tubes to pellet any condensation and transfer 250 µl of each reaction to separate wells in a 96 well plate.
4. Read O.D. with a microplate reader at 540 nm (500-570 nm) immediately.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards and samples.

2.Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

3. Subtract blank OD (S4) from the standard OD values and plot the OD against standard concentrations. Determine the slope using linear regression fitting. The NOS activity of the Sample is then calculated as:

NOS activity (U/L) = [(OD sample - OD sample blank) / Slope] X 1/t

- (OD sample) and (OD sample blank) are optical density values of the sample and sample blank, respectively.

- t is the reaction time (20 min).

- Unit definition: one unit of NOS catalyzes the production of 1 µmole of nitric oxide per minute under the assay conditions (pH 7.5 and 37°C).

QUALITY ASSURANCE

Sensitivity

The minimum detectable dose (MDD) of Nitric Oxide ranged from 0.25 – 25 U/L. The mean MDD was 0.25 U/L.