# Nitric Oxide (NO) Assay Kit

**Catalog No.:** abx294029

**Detection Range**: 1.38 µmol/L – 40 µmol/L

**Sensitivity**: 1.38 µmol/L

**Size:** 96 tests

**Storage:** Store the Enzyme Reagent, Substrate, Chromogenic Reagent A, and Chromogenic Reagent B in the dark at -20°C. Store the rest of the components at -20°C.

**Application:** For detection of Nitric Oxide concentration in serum, plasma, tissue homogenates, cell lysates, urine, and other biological fluids.

**Introduction**

Nitric oxide (NO) is a vital component in many biological functions, including regulation of neurotransmission, blood vessel dilation, and hormone release. Additionally, NO plays a key role in immune response modulation, cardiovascular health and oxygen transport. Excessive NO production can lead to oxidative stress and tissue damage. NO is quickly metabolized into $NO_3$- and $NO_2$- within the body. Nitrate reductase is used to reduce $NO_3$- and $NO_2$-.

Abbexa's Nitric Oxide (NO) Assay Kit is a quick, convenient, and sensitive method for the detection of Nitric Oxide concentration. The product has an absorbance maxima at 530 nm. The intensity of the color is proportional to the Nitric Oxide concentration, which can then be calculated.

**Kit components**

1. 96-well microplate
2. Enzyme Reagent: 4 vials
3. Substrate: 1 vial
4. Sulfate Solution: 3 ml
5. Alkaline Reagent: 1.5 ml
6. Chromogenic Reagent A: 1 vial
7. Chromogenic Reagent B: 1 vial
8. Acid Reagent: 3 ml
9. Standard (1 mmol/L): 2 x 1.5 ml
10. Plate sealer: 2

**Materials Required But Not Provided**

1. Microplate reader (530 nm)
2. Distilled water
3. Normal saline (0.9% NaCl) or PBS (0.01 M, pH 7.4)
4. Pipette and pipette tips
5. Vials/tubes
6. Sonicating water bath
7. Centrifuge
8. Vortex mixer
9. Incubator
10. Ice

## Protocol

### A. Preparation of samples and reagents

#### 1. Reagents

Keep the Enzyme Reagent and Substrate on ice during use. Bring all other reagents to room temperature before use.

- **Enzyme Stock Solution**: Dissolve 1 vial of Enzyme Reagent with 1 ml of distilled water and mix well. Store at 2-8°C in the dark for up to 6 hours.
- **Substrate Working Solution**: Dissolve 1 vial of Substrate with 5 ml of distilled water and mix well. Store at -20°C in the dark for up to 3 days.
- **Enzyme Working Solution**: Mix 30 µl of Enzyme Stock Solution and 30 µl of Substrate Working solution to create 60 µl of Enzyme Working Solution. Prepare 60 µl of Enzyme Working Solution for each well. Prepare just before use. Store at 2-8°C in the dark for up to 6 hours.
- **Chromogenic Reagent A Working Solution**: Dissolve 1 vial of Chromogenic Reagent A with 6 ml of distilled water. Heat in a 90°C water bath to dissolve. Store at 2-8°c in the dark for up to 3 days. When used again, the solution should be heated in a 90°C water bath to dissolve.
- **Chromogenic Reagent B Working Solution**: Dissolve 1 vial of Chromogenic Reagent B in 4 ml of distilled water. Mix well until dissolved. Store at 2-8°C in the dark for up to 3 days.
- **Chromogenic Working Solution**: Mix 50 µl of Chromogenic Reagent A Working Solution, 20 µl of Chromogenic Reagent B Working Solution, and 20 µl of Acid Reagent. Prepare sufficient solution for the amount of wells being used. Prepare just before use and protected from the light.
- **40 µmol/L Standard Solution**: Dilute 168 µl of 1 mmol/L Standard with 4032 µl of distilled water and mix well. Prepare just before use and protected from the light.

#### 2. Samples

Isolate the test samples soon after collecting and analyze immediately or aliquot and store at -20°C or -80°C for long-term storage. Avoid multiple freeze-thaw cycles.

The following sample preparation methods are intended as a guide and may be adjusted as required depending on the specific samples used.

- **Serum:** Samples should be collected into a serum separator tube. Coagulate the serum by leaving the tube undisturbed in a vertical position overnight at 4°C or at room temperature for up to 1 hr. Centrifuge at approximately 2000 × g for 15 mins at 4°C. If a precipitate appears, centrifuge again. Take the supernatant, keep on ice and assay immediately, or aliquot and store at -80°C for up to 1 month.
- **Plasma:** Collect plasma using heparin as the anticoagulant. Centrifuge for 10 mins at 700-1000 × g at 4°C, within 30 mins of collection. If precipitate appears, centrifuge again. Avoid hemolytic samples. Take the supernatant (avoid taking the middle layer containing white blood cells and platelets), keep on ice and assay immediately, or aliquot and store at -80°C for up to 1 month.
- **Tissue Homogenates:** Weigh 40 mg of tissue and wash with pre-chilled PBS. For each 1 mg of tissue, add 9 µl of pre-chilled normal saline (0.9% NaCl). Homogenize by hand, using a mechanical homogenizer, or by

ultrasonication. Centrifuge the homogenate at 1000 x g at 4°C for 10 min. Collect the supernatant and assay immediately. The protein concentration in the supernatant should be determined separately.

- **Cell lysates:** Collect cells into a centrifuge tube and wash with PBS. Add 300 µl of normal saline (0.9% NaCl) or PBS (0.01 M, pH 7.4) per 1 × $10^6$ cells, then sonicate in an ice water bath. Centrifuge at 10,000 × g at 4 °C for 10 min. Take the supernatant into a new centrifuge tube (while kept on ice) and analyze immediately. The protein concentration in the supernatant should be determined separately.
- **Urine:** Collect fresh urine into a sterile container, then centrifuge at 10,000 × g at 4°C for 15 min. Take the supernatant, keep on ice and assay immediately, or aliquot and store at -80°C for up to 1 month.

It is recommended to carry out a preliminary experiment to determine the optimal dilution factor of samples before carrying out the formal experiment.

The recommended dilution factor for different samples is as follows (for reference only):

| Sample Type | Dilution Factor |
|---|---|
| Human Serum | 1 |
| Human Urine | 20-30 |
| Mouse Serum | 1-3 |
| Chicken Plasma | 1 |
| Rat Urine | 20-30 |
| 10% Rat Brain Tissue Homogenate | 1 |
| 10% Rat Kidney Tissue Homogenate | 1 |
| 10% Vegetable Leaf Tissue Homogenate | 1 |
| 1x$10^6$ Jurkat Cell | 1 |

**Note:**

- Fresh samples or recently obtained samples are recommended to prevent degradation and denaturalization that may lead to erroneous results.
- Lysis buffers may interfere with the kit. It is therefore recommended to use mechanical lysis methods for cell lysates and tissue homogenates.
- The diluent is normal saline (0.9% NaCl).



**B. Assay Procedure**

1. Set standard, and sample tubes. Pipette samples up and down to mix before adding to tubes. Avoid foaming or bubbles.
2. Standard curve preparation: Label 8 tubes with 40, 32, 28, 24, 20, 16, 8 and 0 µmol/L. Dilute the 40 µmol/L Standard Solution with distilled water to create 0, 8, 16, 20, 34, 28, 32, 40 µmol/L. See the table for dilution reference.

| **Concentration (μmol/L)** | 0 | 8 | 16 | 20 | 24 | 28 | 32 | 40 |
|---|---|---|---|---|---|---|---|---|
| **40 μmol/L standard (μl)** | 0 | 200 | 400 | 500 | 600 | 700 | 800 | 1000 |
| **Distilled water (μl)** | 1000 | 800 | 600 | 500 | 400 | 300 | 200 | 0 |

3. Aliquot each standard into the corresponding standard tubes (100 μl – 200 μl if using tissue and cell samples, and 80 μl – 100 μl if using serum, plasma and urine samples).
4. Aliquot each sample into the corresponding sample tubes (100 μl – 200 μl if using tissue and cell samples, and 80 μl – 100 μl if using serum, plasma and urine samples). *Note: the same volume of standard and sample should be used.*
5. Add 60 μl of Enzyme Working Solution to each tube. Mix fully and incubate at 37°C in the dark for 60 min.
6. Add 20 μl of Sulfate Solution to each tube.
7. Add 10 μl of Alkaline Reagent to each tube. Mix fully and stand at room temperature for 5 mins.
8. Centrifuge at 10,000 × g for 10 min, then take the supernatant for detection.
9. Set the sample and standard wells on the 96 well microplate and record their positions. We recommend setting up each standard and sample in duplicate.
10. Add 50 μl of Chromogenic Working Solution to each well.
11. Add 120 μl of supernatant from the standard tube to the standard wells. Mix fully.
12. Add 120 μl of supernatant from the sample tube to the sample wells. Mix fully.
13. Stand at room temperature for 5 min.
14. Measure the OD value of each well at 530 nm with a microplate reader.

### C. Calculation of Results

The standard curve can be plotted as the absolute $OD_{530}$ of each standard solution (y) vs. the respective concentration of the standard solution (x). A linear fit is recommended for the standard curve (y = ax + b). Create the standard curve with graph software. The Nitric Oxide concentration of the samples can be interpolated from the standard curve.

**1. Serum, plasma, and urine samples:**

$$\text{NO content (μmol/L)} = \frac{(\Delta A_{530} - b)}{a} \times f$$

**2. Tissues and cell lysate samples:**

$$\text{NO content (μmol/gprot)} = \frac{(\Delta A_{530} - b)}{a} \times \frac{f}{C_{pr}}$$

where:

| | |
|---|---|
| $\Delta A_{530}$ | The absolute OD value of the sample ($\Delta A_{530} = OD_{Sample} - OD_{Blank}$) |
| $C_{pr}$ | The protein concentration of the sample (gprot/L) |
| $f$ | The dilution factor of sample |
| $a$ | Gradient of the standard curve |
| $b$ | Intercept of the standard curve |