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Technical Datasheet

EZAssay™ Nitric Oxide Estimation Kit

Product Code: CCK061

1. Introduction:

Nitric oxide (NO) is produced from L-Arginine by nitric oxide synthase in biological systems. NO is a pleiotropic biological mediator involved in diverse functions such as vasorelaxation by activating soluble guanylate cyclase, inhibition of tumor cells and activities ranging from neuronal function to immune system regulation. Altered levels of NO are indicative of sepsis, hypertension, type II diabetes, hypoxia, cancer etc.

NO is a gaseous free radical which has very short half-life *in vivo* of a few seconds. Therefore, levels of more stable NO metabolites, nitrite (NO_2^-) and nitrate (NO_3^-) are detected spectrophotometrically to estimate NO concentration in biological fluids indirectly.

Reaction

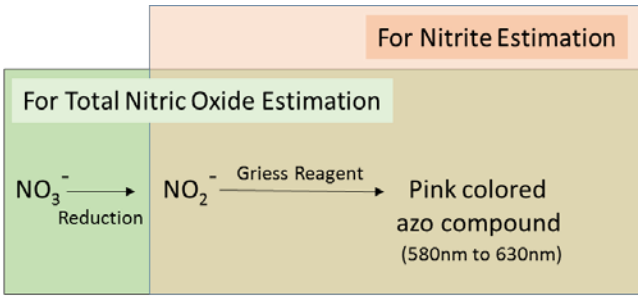
Step I

Nitrate (NO_3^-) —Reduction→ Nitrite (NO_2^-)

Step II

Nitrite (NO_2^-) —Griess Reagent→ Pink colored azo Compound (580nm to 630nm)

Diagrammatic representation of Nitric Oxide Estimation



This assay is based on the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) by a reducing agent at 37^0C. Converted nitrite and endogenous nitrite are collectively converted by Griess reagent to a blue colored azo compound. This compound can be measured spectrophotometrically between 580-630nm and absorbance is directly proportional to the total nitric oxide concentration in the sample which is calculated from standard plot of $NaNO_3$.

Total Nitric Oxide (TNO)

TNO= Nitrite (NO_2^-) + Nitrate (NO_3^-)

Determination of only nitrite (NO_2^-) can be done with the help of standard plot of $NaNO_2$ (only step II).

Determination of Nitrate (NO_3^-) can be done by subtracting Nitrite concentration from Total Nitric oxide concentration.

Determination of only nitrate NO_3^-

Nitrate (NO_3^-) = TNO – Nitrite (NO_2^-)

2. Kit Contents:

Code	Name	Quantity	Storage temp
CCK061(A)	Griess Reagent I	10ml	2 - 8ºC
CCK061(B)	Griess Reagent II	10ml	2 - 8ºC
CCK061(C)	Reducing agent	20ml	2 - 8ºC
CCK061(D)	Nitrite standard	5ml	2 - 8ºC
CCK061(E)	Nitrate standard	5ml	2 - 8ºC

The components of one kit are sufficient to perform 200 tests, including standards.

3. Materials required but not provided in the kit:

- Test sample
- Adjustable pipettes and tips
- 37ºC incubator
- Flat-bottom 96-well microtiter plates
- 96-well microplates reader capable of measuring absorbance between 580-630nm
- Cell Culture Grade Water

4. General Guidelines:

- To obtain statistically significant data, perform the assay in triplicates or more.
- Accuracy of the assay depends on pipetting skills of the personnel. Inappropriate addition and mixing practices may result in erroneous and false-positive or false-negative results.
- Use of a multichannel pipettor is recommended to pipette reagents. This saves time and helps maintain more precise incubation times.
- Pipette tip should be equilibrated with the reagent before use. This is carried out by slowly filling up the tip with reagent and gently expelling the contents several times.
- Care should be taken so that no bubbles are introduced into the wells during pipetting or mixing of the reagents.

5. Directions for use:

Users are advised to review entire procedure before starting the assay

5.1 Sample preparation and measurement:

This kit can be used for estimation of TNO in cell culture supernatant, serum, plasma, urine etc.

5.1.1 Remove the particulate matter from the sample by filtration or centrifugation.

5.1.2 Immediately assay the samples

5.1.3 Otherwise store the samples at -20°C or -80°C depending on the requirement.

5.1.4 Avoid the freeze and thaw cycles.

5.1.5 Dilute the sample from 2 to 20 fold to fall under standard range, while performing assay.

5.2 Determination of Total Nitric Oxide concentration:

Prepare standard curve of Sodium nitrate ($NaNO_3$):

Prepare 320µM sodium nitrate ($NaNO_3$) solution using 200mM $NaNO_3$ (CCK061(E)). For this, add 16µl (CCK061(E)) to 9984µl of cell culture grade water. Serially dilute 320µM $NaNO_3$ in 1:1 ratio to obtain 160 µM, 80µM, 40µM, 20µM, 10µM, 5 µM solution.

For dilution refer **Figure 1**. For assay procedure follow **Table 1**.

Note: If sodium nitrate concentration of sample is too high, either dilute the sample, or prepare standards of higher concentrations. If diluting the sample, multiply by the dilution factor while calculating the final concentration.

Note: The color could get lost if the reaction goes too far due to too long incubation time or too concentrated samples.

Figure 1: Dilution guide for Sodium Nitrate Standards

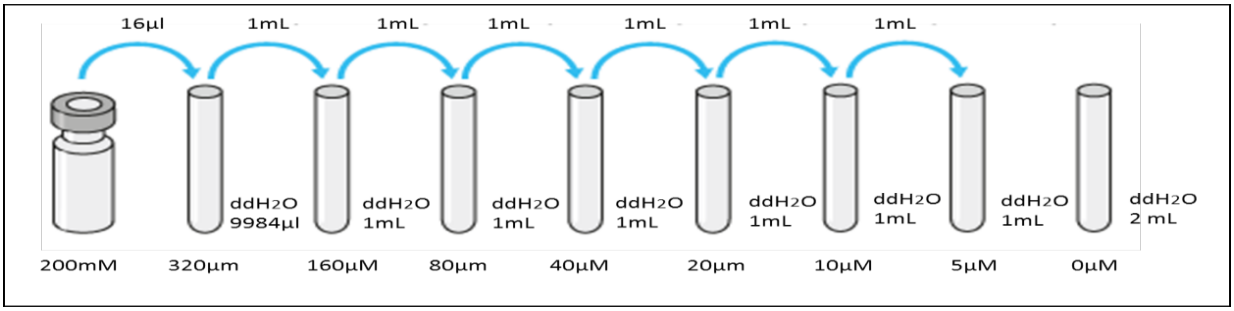


Table 1: Total Nitric Oxide Assay Procedure

$NaNO_3$ (µM)	Water/ Sample/ $NaNO_3$	Volume (µl)	Reducing agent CCK061(C) (µl)	Griess reagent I CCK061(A) (µL)	Griess reagent II CCK061(B) (µL)
0 (Blank)	Water	100	100	50	50
5	$NaNO_3$	100	100	50	50
10	$NaNO_3$	100	100	50	50
20	$NaNO_3$	100	100	50	50
40	$NaNO_3$	100	100	50	50
80	$NaNO_3$	100	100	50	50
160	$NaNO_3$	100	100	50	50
320	$NaNO_3$	100	100	50	50
-	Sample	100	100	50	50
Incubate the plate at 37ºC for 2-4hr and read the absorbance at 580nm (Main) /630nm (Reference).					

5.3 **Determination of endogenous nitrite concentration:**

Prepare standard curve of Sodium nitrite ($NaNO_2$): Prepare 200 µM sodium nitrite ($NaNO_2$) solution using 200mM $NaNO_2$ (CCK061(D)). For this, add 10µl (CCK061(D)) to 9990µl of cell culture grade water. Serially dilute 200µM $NaNO_2$ in 1:1 ratio to obtain 100 µM, 50 µM, 25µM, 12.5µM, 6.25µM, 3.12 µM solution.

For dilution refer **Figure 2**. For assay procedure follow **Table 2**.

Note: If sodium nitrite concentration of sample is too high, either dilute the sample, or prepare standards of higher concentrations. If diluting the sample, multiply by the dilution factor while calculating the final concentration.

Note: The color could get lost if the reaction goes too far due to too long incubation time or too concentrated sample

Figure 2: Dilution guide for Sodium Nitrite Standards

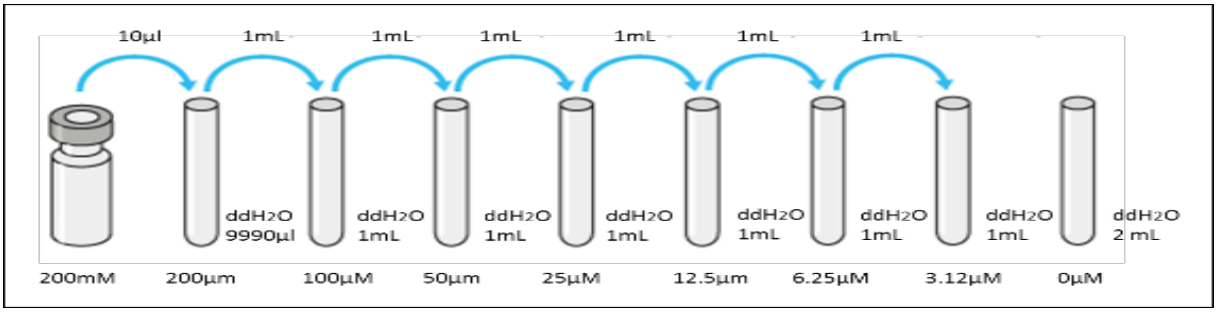
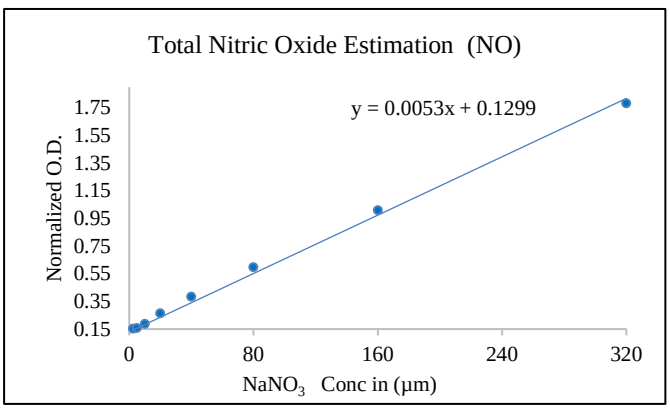


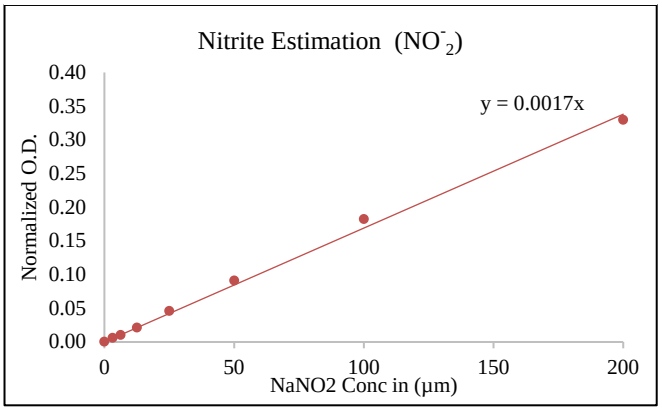
Table 2: $NaNO_2$ Assay Procedure

$NaNO_2$ (µM	Sample/ Water/ $NaNO_2$	Volume (µl)	Griess reagent I CCK061(A) (µL)	Griess reagent II CCK061(B) (µL)
0 (Blank)	Water	100	50	50
3.12	$NaNO_2$	100	50	50
6.25	$NaNO_2$	100	50	50
12.5	$NaNO_2$	100	50	50
25	$NaNO_2$	100	50	50
50	$NaNO_2$	100	50	50
100	$NaNO_2$	100	50	50
200	$NaNO_2$	100	50	50
-	Sample	100	50	50

Graph: 1



Graph: 2



6. Result analysis:

6.1 Determine the average values from duplicate or triplicate readings at 580-630 nm and from this value, subtract the average value of blank. The value obtained is **corrected absorbance**.

Corrected absorbance=

Absorbance $_{(Test/\ standard)}$ **– Absorbance** $_{(580\text{-}630nm)\ (Blank)}$

6.2 Plot the corrected absorbance of standards (Y-axis) against Sodium nitrate concentrations (X-axis) to obtain the standard curve.

6.3 Given below is an example of a standard curve created by following steps described in the assay procedure.
Note: The plot given below is for reference only.
DO NOT use it to interpret actual results.

6.4 Determine slope of the standard curve (y = mx + c).

6.5 Calculate the Total Nitric oxide concentration of each sample using the equation of the standard curve.

$$\text{Total Nitric Oxide } (\mu M) = \frac{(\text{corrected absorbance}) - (\text{y intercept})}{\text{slope}}$$

6.6 Concentration of only nitrite can be determined in a similar fashion as described above.

6.7 To determine the nitrate (NO_3^-) concentration in the sample, the endogenous nitrite (NO_2^-) concentration obtained from nitrite (NO_2^-) assay produce must be subtracted from the total nitric oxide concentration.

7. Storage and Shelf life:

- On receipt, store the kit components at temperatures indicated on individual labels. (Refer section 3).
- Use before the expiry date given on the product label.

8. Advantages:

- **Flexibility**: Different types of samples (serum, cells and tissue) can be analyzed.
- **Compatibility with multiple instruments**: The absorbance can be read using a spectrophotometer or a microplate reader

9. Troubleshooting Points:

Problems	Possible Causes	Recommended Solutions
Random absorbance values / dispersed duplicate and triplicate values	Pipetting errors	Do not splash contents in the tube or cuvette; equilibrate the pipette tips before pipetting each reagent
	Air bubbles formed in the tube/ cuvette/well	Pipette gently against the wall of the tubes/cuvette/well; remove the bubbles by gently tapping the side of the tube/cuvette/well
	Color development solution not prepared using the supplied reagents	Use only the reagents provided in the kit for preparation of color development solution
	Samples used after multiple freeze-thaw cycles	Aliquot the samples before freezing and avoid multiple free-thaw cycles
	Use of old or inappropriately stored samples	Use fresh samples or store at correct temperatures
	Use of reagents diluted previously and stored for long durations	Prepare fresh dilutions of all reagents; refer to datasheet for storage of diluted reagents
	Use of partially thawed samples	Thaw the sample completely. Equilibrate it at room temperature and mix gently before use
Readings do not follow a linear pattern for standard	Colour development solution or dilutions of standard solutions stored for long duration	Prepare these reagents freshly for each assay. Do not store.
	Dilutions of standard stock not prepared correctly	Refer to the datasheet for dilutions of standards; equilibrate the pipette tips while pipetting out standard of each dilution

	Air bubbles formed in the tube/ cuvette/well	Pipette gently against the wall of the tubes/cuvette/well; remove the bubbles by gently tapping the side of the tube/cuvette/well
	Calculation errors	Recheck calculations after referring to the datasheet
	Substituting reagents from older kits/lots	Only use the components given in the kit

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