



**User's Manual**

# **Rat Nox4(Nicotinamide Adenine Dinucleotide Phosphate Oxidase 4) ELISA Kit**



**DEIA-BJ2207**



**96T**



This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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## PRODUCT INFORMATION

### Intended Use

Nox4 ELISA Kit is intended for the in vitro quantitative determination of NOX4 concentrations in serum, plasma, tissue homogenates, cell culture supernatant, cell culture lysate and other biological fluids.

### Principles of Testing

This kit was based on sandwich enzyme-linked immune-sorbent assay technology. Capture antibody was pre-coated onto 96- well plates. And the biotin conjugated antibody was used as detection antibodies. The standards, test samples and biotin conjugated detection antibody were added to the wells subsequently, and washed with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the target amount of sample captured in plate. Read the O.D. absorbance at 450nm in a microplate reader, and then the concentration of target can be calculated.

### Reagents And Materials Provided

|    |                                                                                                                                                                                                                                                                                                                                                         |
|----|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1  | <b>Dilute each of the standards and samples (serum/plasma) at 1:100 using Assay Buffer</b> as described in "Dilution of Standards and Samples (serum/plasma)" section.                                                                                                                                                                                  |
| 2  | Pipette <b>100µl</b> of <b>Assay Buffer</b> non-exceptionally into each of the wells to be used.                                                                                                                                                                                                                                                        |
| 3  | Pipette <b>25 µL</b> of <b>Diluted Standards, High Level Control, Low Level control and Samples</b> into the respective wells of microtiter plate.<br><b>Wells</b><br>A1: Standard A<br>B1: Standard B<br>C1: Standard C<br>D1: Standard D<br>E1: Standard E<br>F1: High Level Control<br>G1: Low Level Control<br>H1 and on: Sample ( Serum / Plasma ) |
| 4  | Cover the plate with adhesive film. <b>Incubate 30 min</b> at room temperature (18-25°C).                                                                                                                                                                                                                                                               |
| 5  | Remove adhesive film. Discard incubation solution. Wash plate <b>3 times</b> each with <b>300 µL</b> of diluted <b>Wash Buffer</b> . Remove excess solution by tapping the inverted plate on a paper towel.                                                                                                                                             |
| 6  | Pipette <b>100 µL</b> of ready-to use <b>HRP Conjugate</b> into each well.                                                                                                                                                                                                                                                                              |
| 7  | Cover the plate with a new adhesive film. <b>Incubate 30 min</b> at room temperature                                                                                                                                                                                                                                                                    |
| 8  | Remove adhesive foil. Discard incubation solution. Wash plate 3 times each with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.                                                                                                                                                                   |
| 9  | Pipette 100 µL of TMB Substrate Solution into each well.                                                                                                                                                                                                                                                                                                |
| 10 | <b>Incubate 10 min</b> (without adhesive film) at room temperature (18-25°C) in the dark.                                                                                                                                                                                                                                                               |
| 11 | Stop the substrate reaction by adding <b>100 µL</b> of <b>Stop Solution</b> into each well. Briefly mix contents by gently shaking the plate. Color changes from blue to yellow.                                                                                                                                                                        |
| 12 | <b>Measure</b> optical density with a photometer at <b>450/650 nm</b> within <b>30 min</b> after pipetting of the Stop Solution.                                                                                                                                                                                                                        |



## Materials Required But Not Supplied

1. Microplate reader (wavelength:450nm)
2. 37°C incubator
3. Automated plate washer
4. Precision single and multi-channel pipette and disposable tips
5. Clean tubes and Eppendorf tubes
6. Deionized or distilled water

## Storage

2-8 °C for 6 months.

## Specimen Collection And Preparation

### Serum:

Place whole blood sample at room temperature for 2 hours or put it at 2-8°C overnight and centrifugation for 20 minutes at approximately 1000×g. Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.

### Plasma:

Collect plasma using EDTA-Na<sub>2</sub> or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2-8°C within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Avoid hemolysis, high cholesterol samples.

### Tissue Homogenates:

As hemolysis blood has relation to assay result, it is necessary to remove residual blood by washing tissue with pre-cooling PBS buffer (0.01M, pH=7.4). Mince tissue after weighing it and get it homogenized in PBS (the volume depends on the weight of the tissue. Normal, 9mL PBS would be appropriate to 1 gram tissue pieces. Some protease inhibitors are recommended to add into the PBS) with a glass homogenizer on ice. To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 minutes at 5000×g to get the supernatant. The total protein concentration was determined by BCA kit and the total protein concentration of each pore sample should not exceed 0.3mg.

### Cell Culture Supernatant:

Centrifuge supernatant for 20 minutes at 1000×g at 2-8°C to remove insoluble impurity and cell debris. Collect the clear supernatant and carry out the assay immediately.

### Cell Culture Lysate:

Commercial RIPA kits are recommended to follow the instructions provided. Generally, 0.5ml RIPA lysis buffer would be appropriate to 2×10<sup>6</sup> cells, DNA must to be removed. The total protein concentration was determined by BCA kit and the total protein concentration of each pore sample should not exceed 0.3mg.

### Other Biological Fluids:

Centrifuge samples for 20 minutes at 1000×g at 2-8°C. Collect supernatant and carry out the assay immediately.

**Note:**

Samples to be used within 5 days can be stored at 2-8°C, besides that, samples must be stored at -20°C (assay ≤1 month) or -80°C (assay ≤2 months) to avoid loss of bioactivity and contamination. Avoid multiple freeze-thaw cycles. The hemolytic samples are not suitable for this assay.

**Sample Dilution:**

The user should estimate the concentration of target protein in the test sample, and select a proper dilution factor to make the diluted target protein concentration fall in the optimal detection range of the kit. Dilute the sample with the provided dilution buffer, and several trials may be necessary. The test sample must be well mixed with the dilution buffer. And also standard curves and sample should be making in pre-experiment. If samples with very high concentrations, dilute samples with PBS first and then dilute the samples with Sample Dilution.

**The matrix components in the sample will affect the test results, which it need to be diluted at least 1/2 with Sample Dilution Buffer before testing!**

**Reagent Preparation**

Bring all reagents and samples to room temperature for 20 minutes before use.

**1. Wash Buffer:**

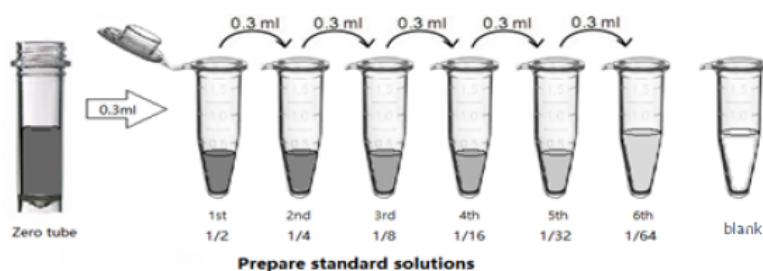
If crystals have formed in the concentrate, you can warm it with 40°C water bath (Heating temperature should not exceed 50°C) and mix it gently until the crystals have completely been dissolved. The solution should be cooled to room temperature before use. Dilute 30ml Concentrated Wash Buffer into 750ml Wash Buffer with deionized or distilled water. Put unused solution back at 2-8°C.

**2. Standards:**

- 1) Add 1 ml Sample Dilution Buffer into one Standard tube (labeled as zero tube), keep the tube at room temperature for 10 minutes and mix them thoroughly.

**Note: If the standard tube concentration higher than the range of the kit, please dilute it and labeled as zero tube.**

- 2) Label 7 EP tubes with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank respectively. Add 0.3ml of the Sample Dilution Buffer into each tube. Add 0.3ml of the above Standard solution (from zero tube) into 1<sup>st</sup> tube and mix them thoroughly. Transfer 0.3ml from 1<sup>st</sup> tube to 2<sup>nd</sup> tube and mix them thoroughly. Transfer 0.3ml from 2<sup>nd</sup> tube to 3<sup>rd</sup> tube and mix them thoroughly, and so on. Sample Dilution Buffer was used for the blank control.



**Note:**

It is best to use Standard Solutions within 2 hours.

**3. Preparation of Biotin-labeled Antibody Working Solution:**

Prepare it within 1 hour before experiment.

- 1) Calculate required total volume of the working solution:  $0.1\text{ml/well} \times \text{quantity of wells}$ . (Allow 0.1-0.2ml more than the total volume.)
- 2) Dilute the Biotin-detection antibody with Antibody Dilution Buffer at 1:100 and mix them thoroughly. (i.e. Add 1ul Biotin-labeled antibody into 99ul Antibody Dilution Buffer.)

**4. Preparation of HRP-Streptavidin Conjugate (SABC) Working Solution:**

Prepare it within 30 minutes before experiment.

- 1) Calculate required total volume of the working solution:  $0.1\text{ml/well} \times \text{quantity of wells}$ . (Allow 0.1-0.2ml more than the total volume.)
- 2) Dilute the SABC with SABC Dilution Buffer at 1:100 and mix them thoroughly. (i.e. Add 1ul of SABC into 99ul of SABC Dilution Buffer.)

**Assay Procedure****Washing Notes**

**Manual:** Discard the solution in the plate without touching the side walls. Clap the plate on absorbent filter papers or other absorbent material. Fill each well completely with 350ul wash buffer and soak for 1 to 2 minutes, then aspirate contents from the plate, and clap the plate on absorbent filter papers or other absorbent material.

**Automatic:** Aspirate all wells, and then wash plate with 350ul wash buffer. After the final wash, invert plate, and clap the plate on absorbent filter papers or other absorbent material. It is recommended that the washer shall be set for soaking 1 minute. (**Note:** set the height of the needles; be sure the fluid can be sipped up completely)

**Procedure**

When diluting samples and reagents, they must be mixed completely and evenly. Before adding TMB into wells, equilibrate TMB Substrate for 30 minutes at 37°C. It is recommended to plot a standard curve for each test.

1. Set standard, **test samples (diluted at least 1/2 with Sample Dilution Buffer)**, control (blank) wells on the pre-coated plate respectively, and then, records their positions. It is recommended to measure each standard and sample in duplicate. **Wash plate 2 times before adding standard, sample and control (blank) wells!**
2. **Prepare Standards:** Aliquot 100ul of zero tube, 1<sup>st</sup> tube, 2<sup>nd</sup> tube, 3<sup>rd</sup> tube, 4<sup>th</sup> tube, 5<sup>th</sup> tube, 6<sup>th</sup> tube and Sample Dilution Buffer (blank) into the standard wells.
3. **Add Samples:** Add 100ul of properly diluted sample into test sample wells.
4. **Incubate:** Seal the plate with a cover and incubate at 37°C for 90 minutes.
5. **Wash:** Remove the cover and discard the plate content, and wash plate 2 times with Wash Buffer. Do NOT let the wells dry completely at any time.

6. **Biotin-labeled Antibody:** Add 100ul Biotin-labeled antibody working solution into above wells (standard, test sample and blank wells). Add the solution at the bottom of each well without touching the sidewall, cover the plate and incubate at 37°C for 60 minutes.
7. **Wash:** Remove the cover, and wash plate 3 times with Wash Buffer, and let the Wash Buffer stay in the wells for 1-2 minutes each time.
8. **HRP-Streptavidin Conjugate (SABC):** Add 100ul of SABC Working Solution into each well, cover the plate and incubate at 37°C for 30 minutes.
9. **Wash:** Remove the cover and wash plate 5 times with Wash Buffer, and let the wash buffer stay in the wells for 1-2 minutes each time.
10. **TMB Substrate:** Add 90ul TMB Substrate into each well, cover the plate and incubate at 37°C in dark within 10-20 minutes. (**Note:** The reaction time can be shortened or extended according to the actual color change, but not more than 30 minutes. You can terminate the reaction when apparent gradient appeared in standard wells.)
11. **Stop:** Add 50ul Stop Solution into each well. The color will turn yellow immediately. The adding order of Stop Solution should be as the same as the TMB Substrate Solution.
12. **OD Measurement:** Read the O.D. absorbance at 450nm in Microplate Reader immediately after adding the stop solution.

## Calculation

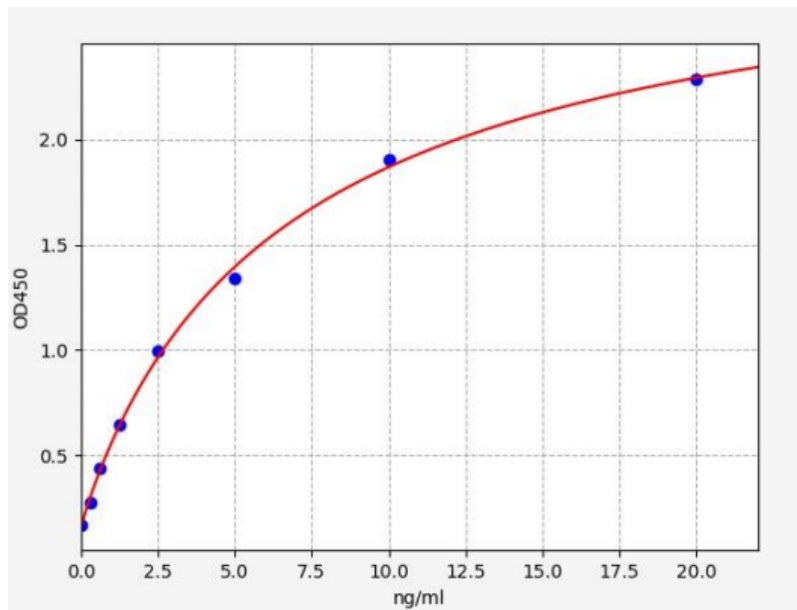
Regarding calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of blank well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The target concentration of the samples can be interpolated from the standard curve. It is recommended to use some professional software to do this calculation.

**Note:** If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

## Typical Standard Curve

Results of a typical standard operation of a Nox4 ELISA Kit are listed below. This standard curve was generated at our lab for demonstration purpose only. Users shall obtain standard curve as per experiment by themselves. (N/A=not applicable)

| STD.(ng/ml) | OD-1  | OD-2  | Average | Corrected |
|-------------|-------|-------|---------|-----------|
| 0           | 0.166 | 0.17  | 0.168   | 0.000     |
| 0.312       | 0.271 | 0.279 | 0.275   | 0.107     |
| 0.625       | 0.433 | 0.445 | 0.439   | 0.271     |
| 1.25        | 0.639 | 0.657 | 0.648   | 0.48      |
| 2.5         | 0.984 | 1.012 | 0.998   | 0.83      |
| 5           | 1.321 | 1.359 | 1.34    | 1.172     |
| 10          | 1.878 | 1.932 | 1.905   | 1.737     |
| 20          | 2.253 | 2.319 | 2.286   | 2.118     |



## Precision

Intra-Assay: CV<8%

Inter-Assay: CV<10%

## Detection Range

0.313-20ng/ml

## Sensitivity

0.188ng/ml

## Specificity

This assay has high sensitivity and excellent specificity for detection of Nox4. No significant cross-reactivity or interference between Nox4 and analogues was observed.

Note: Limited by current skills and knowledge, it is difficult for us to complete the cross-reactivity detection between Nox4 and all the analogues, therefore, cross reaction may still exist.

## Linearity

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of Nox4 and their serial dilutions. The results were demonstrated by percentage of calculated concentration to the expectation.

| Sample              | 1:2    | 1:4     | 1:8     |
|---------------------|--------|---------|---------|
| Serum(n=5)          | 86-99% | 86-103% | 85-100% |
| EDTA Plasma(n=5)    | 82-96% | 84-98%  | 82-97%  |
| Heparin Plasma(n=5) | 85-97% | 81-100% | 85-99%  |

## Recovery

Matrices listed below were spiked with certain level of Nox4 and the recovery rates were calculated by comparing the measured value to the expected amount of Nox4 in samples.

| Matrix              | Recovery Range (%) | Average (%) |
|---------------------|--------------------|-------------|
| Serum(n=5)          | 91-104             | 97          |
| EDTA Plasma(n=5)    | 87-98              | 91          |
| Heparin Plasma(n=5) | 87-105             | 94          |

## Precautions

1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, pilot experiment using standards and a small number of samples is recommended.
2. After opening and before using, keep plate dry.
3. Before using the kit, spin tubes and bring down all components to the bottom of tubes.
4. Storage TMB reagents avoid light.
5. Washing process is very important, not fully wash easily cause a false positive and high background.
6. Duplicate well assay is recommended for both standard and sample testing.
7. Don't let microplate dry at the assay, for dry plate will inactivate active components on plate.
8. Don't reuse tips and tubes to avoid cross contamination.
9. Avoid using the reagents from different batches together.