AssayGenie

Technical Manual

Rat NADPH oxidase 4 (Nox4) ELISA Kit

- **Catalogue Code: RTEB1005**
- **Sandwich ELISA Kit**
- **Research Use Only**

Contents

1. Description and Principle 3
2. Key features and Sample Types 3
3. Workflow Overview 4
4. Kit Components 5
5. Shipping and Storage 6
6. Sample Preparation 6
7. Protocol 7
8. Data analysis 10
9. Important General Notes 11

1. Description and Principle

The Assay Genie Rat NADPH oxidase 4 (Nox4) ELISA Kit Assay can assay for Rat NADPH oxidase 4 (Nox4) in the following samples: serum, blood, plasma, cell culture supernatant and other related supernatants and tissues.

How Do Our ELISA Kit Assays Work?

The Assay Genie ELISA (enzyme-linked immunosorbent assays) assay kits are designed for the quantitative measurement of analytes in a wide variety of samples. As today's scientists demand high quality consistent data for high impact journals, ELISA Genie have developed the range of sensitive, fast, and reliable ELISA Kit Assays to meet and exceed those demands. Our assay kits use a quantitative sandwich ELISA technique, and each kit comes with highly specific antibodies pre-coated on a 96-well microtiter plate.

At Assay Genie we understand the need for speed! Therefore, we have developed an ultra-fast protocol meaning you achieve your results rapidly. This ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Rat NADPH oxidase 4 (Nox4). Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Rat NADPH oxidase 4 (Nox4) and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. The plate is washed to remove all non-specific binding that may have occurred. The substrate solution is added to each well. Only those wells that contain Rat NADPH oxidase 4 (Nox4), biotinylated detection antibody and Avidin-HRP conjugate will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the change in the optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Rat NADPH oxidase 4 (Nox4). You can calculate the concentration of Rat NADPH oxidase 4 (Nox4) in the samples by comparing the OD of the samples to the standard curve.

2. Key features and Sample Types

Compatible with routine laboratory and HTS formats: assays can be performed in microplates.

Assay range: 0.312-20ng/mL

Sensitivity: 0.16ng/mL

Sample types: serum, plasma, saliva, urine, cell culture supernatant, tissue samples and other related supernatants.

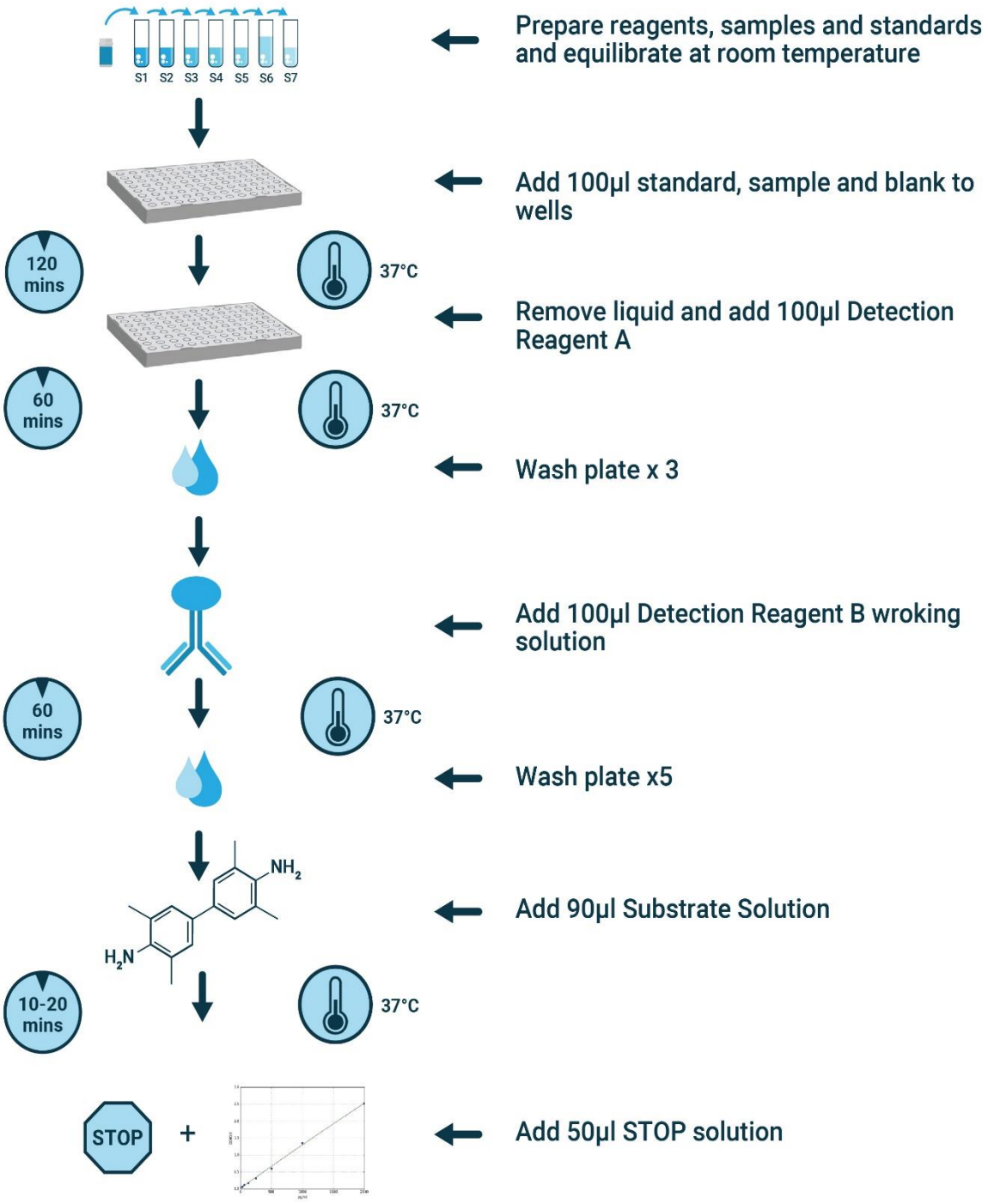
Assay type: Sandwich

Performance Characteristics: Intra-Assay CV: **6.1%**

Inter-Assay CV: **8.4%**

Spike Average Recovery: **(Provided with kit)**

3. Workflow Overview



4. Kit Components

Each kit contains reagents for 48/96 assays in a 96 well plate including:

No.	Component	48 assays	96 assays	Storage
1.	Assay Plate	1	1	Stored at -20°C
2.	Standard	1 vial	2 vials	
3.	Sample Diluent	1 x 10 mL	1 x 20 mL	
4.	Assay Diluent A	1 x 5 mL	1 x 10 mL	
5.	Assay Diluent B	1 x 5 mL	1 x 10 mL	
6.	Detection Reagent A	1 x 60 µL	1 x 120 µL	
7.	Detection Reagent B	1 x 60 µL	1 x 120 µL	
8.	Wash Buffer (25 x concentrate)	1 x 15 mL	1 x 30 mL	Stored at 4°C
9.	Substrate	1 x 5 mL	1 x 10 mL	
10.	Stop Solution	1 x 5 mL	1 x 10 mL	
11.	Plate Sealers	3 pieces	5 pieces	
12.	Manual	1	1	

Additional Materials required:

1. 37ºC incubator
2. Plate shaker
3. Plate Reader with 450nm filter
4. Precision pipettes and disposable pipette tips
5. Distilled water
6. Disposable tubes for sample dilution
7. Absorbent paper

5. Shipping and Storage

The **Assay Plate, Standard, Detection Reagent A and Detection Reagent B** should be stored at -20ºC upon being received. After receiving the kit, the **Substrate should be stored at 4ºC**. Other reagents are kept according to the labels on vials. But for long term storage, please keep the whole kit at -20ºC (Except the substrate). The unused strips should be kept in a sealed bag with the desiccant provided to minimize exposure to damp air. The test kit may be used throughout the expiration date of the kit (six months from the date of manufacture). Opened test kits will remain stable until the expiring date shown, provided it is stored as prescribed above.

6. Sample Preparation

General considerations: extract samples as soon as possible after specimen collection. For best results, experiments should be carried out as soon as possible after the extraction. Alternatively, extract can be kept at -20ºC but for optimal results, avoid repeated freeze-thaw cycles. Samples that contain NaN3 cannot be detected as it interferes with HRP.

Serum: Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 × g. Remove serum and assay immediately or aliquot and store samples at -20ºC or -80ºC.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000 × g at 2ºC - 8ºC within 30 minutes of collection. Store samples at -20ºC or -80ºC. Avoid repeated freeze-thaw cycles. Note: over hemolyzed samples are not suitable for use with this kit.

Urine: collect in a sterile container, centrifuge for 20 mins @ 2000-3000 rpm. Remove supernatant and if any precipitation is detected repeat centrifugation step. A similar protocol can be used for cerebrospinal fluid.

Cell culture supernatant: collect supernatant and centrifuge @ 4ºC for 20 mins @ 2000-3000 rpm. Remove supernatant and rinse cells x2 times with PBS (pH 7.2-7.4) and perform a total cell count. Optimal cell concentration is 1 million / ml. To release cellular components, dilute the cell pellet in PBS and use 3-4 freeze-thaw cycles in liquid Nitrogen (commercial lyses buffers can be used according to manufacturer's instructions). Centrifuge @ 4ºC for 20 mins @ 2000-3000 rpm to pellet debris and remove clear supernatant to clean microcentrifuge tube for ELISA or storage.

Tissue samples: the preparation of tissue homogenates will vary depending upon tissue type. For this assay, tissue was rinsed with 1X PBS to remove excess blood, homogenized in 20mL of 1X PBS and stored overnight at ≤ -20ºC. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g. Remove the supernatant and assay immediately or aliquot and store at ≤ -20ºC.

Notes:

1. Samples to be used within 5 days may be stored at 2-8ºC, otherwise samples must be stored at -20ºC (1 month) or -80ºC (2 months) to avoid loss of bioactivity and contamination.
2. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.
3. Cell viability, cell number, sampling time and samples from cell culture supernatant may influence detection by the kit.
4. Sample hemolysis will influence the result, so hemolyzed samples cannot be detected.
5. When performing the assay slowly bring samples to room temperature.

7. Protocol

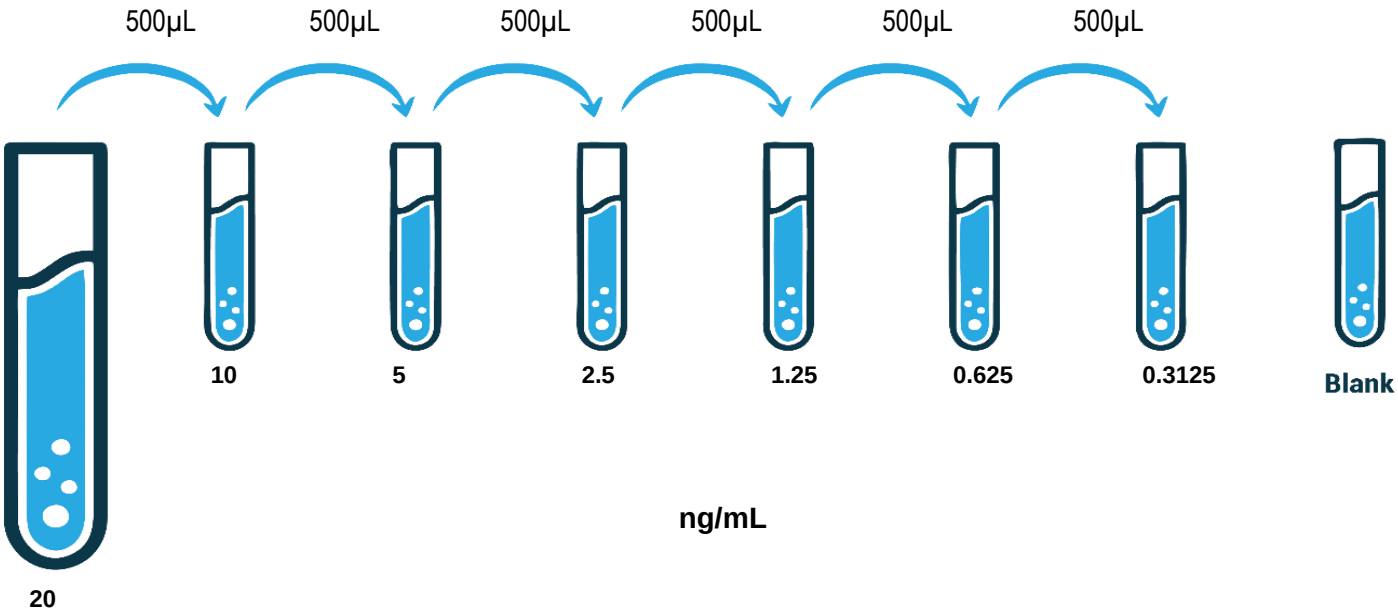
Important: After removal from storage at 2-8ºC, the kit should be equilibrated for 30 minutes at ambient temperature before use. If the coated plates haven't been used after opening, the remaining plates should be stored in the sealed bag.

Procedure using 96-well plate:

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 30 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 750 mL of Wash Buffer.

Standard dilution - Prepare standard within 15 minutes before use. Centrifuge at 10,000×g for 1 minute and reconstitute the Standard with **(2.0/1.0/0.5 mL)** of Sample Diluent. Tighten the lid, let it stand for 15 minutes and invert tube several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a stock solution 20 ng/mL. Make serial dilutions as needed (making serial dilution in the wells directly is not recommended). The undiluted stock serves as the standard with the highest concentration 20 ng/mL. The Sample Diluent serves as the zero blank (0 ng/mL). (Standards can also be diluted according to the actual amount, such as 200µL/tube).

10 ng/mL	Standard 2	500µl Original Standard + 500µl Sample Diluent
5 ng/mL	Standard 3	500µl Standard 2 + 500µl Sample Diluent
2.5 ng/mL	Standard 4	500µl Standard 3 + 500µl Sample Diluent
1.25 ng/mL	Standard 5	500µl Standard 4 + 500µl Sample Diluent
0.625 ng/mL	Standard 6	500µl Standard 5 + 500µl Sample Diluent
0.3125 ng/mL	Standard 7	500µl Standard 6 + 500µl Sample Diluent
0	Blank	500µl Sample Diluent



Detection Reagent A and B - Dilute to the working concentration using Assay Diluent A and B (1:100), respectively.

Washing Procedure:

1. **Automated Washer:** Add 400µL wash buffer into each well, the interval between injection and suction should be set about 60s.

2. **Manual wash:** Add 400µL Wash Buffer into each well, soak it for 1~2minutes. After the last wash, decant any remaining Wash Buffer by inverting the plate and blotting it dry by rapping it firmly against clean absorbent paper on a hard surface.

Assay procedure:

Allow all reagents to reach room temperature (Please do not dissolve the reagents at 37ºC directly). **All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming.** Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Removed strips should be resealed and stored at -20ºC until the kits expiry date. Prepare all reagents, working standards and samples as directed in the previous sections. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their experiments. We recommend running all samples in duplicate.

1. **Add Sample:** Add 100µL of Standard, Blank, or Sample per well. The blank well is added with Sample diluent. Solutions are added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible. Mix it gently. Cover the plate with sealer we provided. Incubate for 120 minutes at 37ºC.

2. **Detection Reagent A:** Remove the liquid from each well, don't wash. Add 100µL of Detection Reagent A working solution to each well. Cover with the Plate sealer. Gently tap the plate to ensure thorough mixing. Incubate for 1 hour at 37°C. Note: if Detection Reagent A appears cloudy warm to room temperature until solution is uniform.

3. **Wash:** Aspirate each well and wash, repeating the process three times. Wash by filling each well with Wash Buffer (approximately 400µL) (a squirt bottle, multi-channel pipette, manifold dispenser or automated washer are needed). Complete removal of liquid at each step is essential. After the last wash, completely remove remaining Wash Buffer by aspirating or decanting. Invert the plate and pat it against thick, clean absorbent paper.

4. **Detection Reagent B:** Add 100µL of **Detection Reagent B** working solution to each well. Cover with the Plate sealer. Incubate for 60 minutes at 37°C.

5. **Wash:** Repeat the wash process for five times as conducted in step 3.

6. **Substrate:** Add 90µL of Substrate Solution to each well. Cover with a new Plate sealer and incubate for 10-20 minutes at 37°C. Protect the plate from light. The reaction time can be shortened or extended according to the actual color change, but this should not exceed more than 30 minutes. When apparent gradient appears in standard wells, user should terminate the reaction.

7. **Stop**: Add 50µL of Stop Solution to each well. If the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

8. **OD Measurement**: Determine the optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm. User should open the micro-plate reader in advance, preheat the instrument, and set the testing parameters.

9. After experiment, store all reagents according to the specified storage temperature respectively until their expiry.

Additional Information on protocol

1. Absorbance is a function of the incubation time. Therefore, prior to starting the assay it is recommended that all reagents should be freshly prepared prior to use and all required strip-wells are secured in the microtiter frame. This will ensure equal elapsed time for each pipetting step, without interruption.

2. Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction and avoid foaming and mix gently until the crystals have completely dissolved. **The reconstituted Standards Detection Reagent A and B can be used only once.** This assay requires pipetting of small volumes. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated.

3. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay.

4. For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 10 minutes.

5. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

6. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

7. Duplication of all standards and specimens, although not required, is recommended.

8. Substrate Solution is easily contaminated. Please protect it from light.

9. If samples generate values higher than the highest standard, further dilute the samples with the Sample Diluent and repeat the assay. Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time, temperature or kit age can cause variation in binding.

8. Data analysis

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four-parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the Rat NADPH oxidase 4 (Nox4) concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. It is recommended to use some related software to do this calculation, such as curve expert 1.3. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

9. Important General Notes

1. The final experimental results will be closely related to validity of the products, laboratory skills of the end-users and the experimental conditions. Please make sure that sufficient samples are available.

2. Kits from different batches may be a little different in detection range, sensitivity and color developing time. Please perform the experiment exactly according to the instructions.

3. There may be some foggy substance in the wells when the plate is opened at the first time. It will not have any effect on the final assay results.

4. Do not remove microtiter plate from the storage bag until needed.

5. A microtiter plate reader with a bandwidth of 10nm or less and an optical density range of 0-3 OD or greater at 450nm wavelength is acceptable for use in absorbance measurement.

6. Use fresh disposable pipette tips for each liquid transfer to avoid contamination.

7. Do not substitute reagents from one kit lot to another. Use only the reagents supplied by the manufacturer.

8. To achieve reproducible results, the operation of every step in the assay should be controlled. Furthermore, a preliminary experiment before every assay for each batch is recommended.

9. Each kit has been strictly passed Q.C tested. However, results from end-users might be inconsistent with our in-house data due to some unexpected transportation conditions or different lab equipment. Intra-assay variance among kits from different batches might arise from factors.

Safety Precaution

1. The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

Assay Genie 100% money-back guarantee!

If you are not satisfied with the quality of our products and our technical team cannot resolve your problem, we will give you 100% of your money back.

Contact Details

Email: info@ASSAYGenie.com

Web: www.ASSAYGenie.com