

3-Nitrotyrosine ELISA Kit

(Catalog # K4158-100, 100 assays, Store at 4°C)

rev 06/19

I. Introduction:

3-Nitrotyrosine (3-NT) is a biomarker of nitrogen free radical species modified proteins in systemic autoimmune conditions. This ELISA kit uses Competitive-ELISA as the method. The microtiter plate provided in this kit has been pre-coated with 3-NT. During the reaction 3-NT in the sample or standard competes with a fixed amount of 3-NT on the solid phase supporter for sites on the Biotinylated Detection Antibody specific to 3-NT. Excess conjugate and unbound sample or standard are washed from the plate, and HRP-Streptavidin (SABC) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of 3-NT in the samples is then determined by comparing the O.D. of the samples to the standard curve.

II. Application:

This ELISA kit is used for *in vitro* quantitative determination of 3-Nitrotyrosine.

III. Sample Type:

Serum, plasma, tissue homogenates, cell culture supernatant and other biological fluids.

IV. Kit Contents:

Components	K4158-100	Part No.
Micro ELISA Plate	8 X 12 strips	K4158-100-1
Lyophilized Standard	2 vials	K4158-100-2
Sample / Standard dilution buffer	20 ml	K4158-100-3
Biotin- detection antibody (Concentrated)	60 µl	K4158-100-4
Antibody dilution buffer	10 ml	K4158-100-5
HRP-Streptavidin Conjugate (SABC)	120 µl	K4158-100-6
SABC dilution buffer	10 ml	K4158-100-7
TMB substrate	10 ml	K4158-100-8
Stop Solution	10 ml	K4158-100-9
Wash buffer (25X)	30 ml	K4158-100-10
Plate sealers	5	K4158-100-11

V. User Supplied Reagents and Equipment:

- Microplate reader capable of measuring absorbance at 450 nm
- 37°C incubator
- Precision pipettes with disposable tips.
- Distilled or deionized water
- Clean eppendorf tubes for preparing standards or sample dilutions
- Absorbent paper

VI. Storage and Handling:

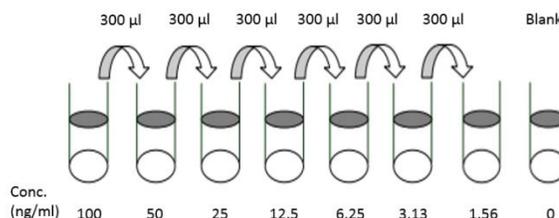
The entire kit may be stored at 4°C for up to 6 months from the date of shipment. Avoid freeze-thaw cycles.

VII. Reagent Preparation:

Note: Prepare reagents within 30 minutes before the experiment.

Bring all reagents to room temperature before use.

- Biotin- detection antibody working solution:** Calculate the total volume of the working solution: 0.05 ml / well x quantity of wells with 0.1 - 0.2 ml more than the total volume. Dilute the Biotin- detection antibody with Antibody dilution buffer at 1:100 and mix thoroughly.
- HRP-Streptavidin Conjugate (SABC):** Calculate the total volume of the working solution: 0.1 ml / well x quantity of wells with 0.1 - 0.2 ml more than the total volume. Dilute the SABC with SABC dilution buffer at 1:100 and mix thoroughly.
- Wash Buffer:** Dilute 30 mL of Concentrated Wash Buffer into 750 mL of deionized or distilled water. Put unused solution back at 4°C. 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 dissolved. The solution should be cooled to room temperature before use.
- Standard Preparation:**
 - Reconstitute the lyophilized Human 3-Nitrotyrosine standard by adding 1 ml of Standard/Sample Dilution Buffer to make the 100 ng/ml standard stock solution.
 - Allow solution to sit at room temperature for 10 minutes, then gently vortex to mix completely. Use within 2 hours of reconstituting.
 - Prepare 0.6 ml of 5 ng/ml top standard by adding 0.3 ml of the above stock solution in 0.3 ml of Standard/Sample Dilution Buffer. Perform 2-fold serial dilutions of the top standards to make the standard curve within the range of this assay.



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- Suggested standard points are: 50, 25, 12.5, 6.25, 3.13, 1.56, 0 ng/ml

5. Sample Preparation:

Note: Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination. Homogenized samples are not suitable for use in this assay.

- **Serum:** Allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000xg. 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-Na₂ as an anticoagulant. Centrifuge samples for 15 minutes at 1000xg at 4°C within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Avoid hemolysis, high cholesterol samples.
- **Tissue homogenates:** For general information, hemolysis blood may affect the result, so you should rinse the tissues with ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then minced to small pieces which will be homogenized in PBS (the volume depends on the weight of the tissue. 9mL PBS would be appropriate to 1 gram tissue pieces. Some protease inhibitor is 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 5000xg to get the supernatant.
- **Cell culture supernatant:** Centrifuge supernatant for 20 minutes to remove insoluble impurity and cell debris at 1000xg at 2 - 8°C. Collect the clear supernatant and carry out the assay immediately.
- **Other biological fluids:** Centrifuge samples for 20 min at 1000xg at 4°C. Collect the supernatant and carry out the assay immediately.
- End user should estimate the concentration of the target protein in the test sample first, and select a proper dilution factor to make the diluted target protein concentration falls the optimal detection range of the kit.

VIII. Assay Protocol:

Note: Bring all reagents and samples to room temperature before use.

It is recommended that all standards and samples be run at least in duplicate.

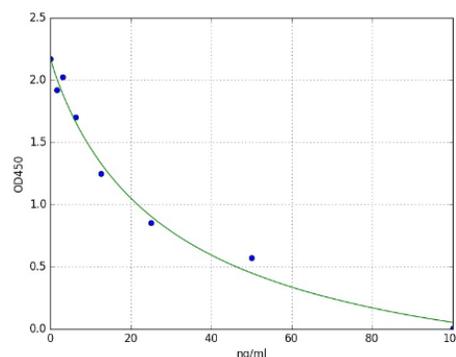
A standard curve must be run with each assay.

1. Prepare all reagents, samples and standards as instructed in section VII.
2. Wash plate 2 times with **1X Wash Solution** before adding standard, sample and control wells.
3. Add 50 µl of each **standard** and **samples** into appropriate wells. Immediately add 50 µL of **Biotinylated Detection Antibody working solution** to each well.
4. Cover well and gently tap the plate to ensure thorough mixing. Incubate for 45 minutes at 37°C.
5. Discard the solution and wash 3 times with **1X Wash Solution**. Wash by filling each well with Wash Buffer (350 µl) using a multi-channel Pipette or autowasher. Let it soak for 1 to 2 minutes and then all residual wash-liquid must be drained from the wells by aspiration. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. **HRP-Streptavidin Conjugate (SABC):** Add 100µL of SABC working solution to each well. Cover with a new Plate sealer. Incubate for 30 minutes at 37°C.
7. Discard the solution and wash 3 times with **1X Wash Solution**. Wash by filling each well with Wash Buffer (350 µl) using a multi-channel Pipette or autowasher. Let it soak for 1 to 2 minutes and then all residual wash-liquid must be drained from the wells by aspiration. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
8. **TMB Substrate:** Add 90µL of TMB Substrate to each well. Cover with a new Plate sealer. Incubate for about 15 minutes at 37°C. Protect from light. The reaction time can be shortened or extended according to the actual color change, but not more than 30 minutes. When apparent gradient appeared in standard wells, you can terminate the reaction.
9. Add 50 µl of **Stop Solution** to each well. Color turn to yellow immediately. The adding order of stop solution should be as the same as the substrate solution. Read result at 450 nm within 20 minutes.

IX. CALCULATION:

Average the duplicate readings for each standard and samples. Create a standard curve by plotting the mean OD Value for each standard on the y-axis or x-axis against the concentration on the x-axis or y-axis and draw a best fit curve through the points on the graph. Generate a best fitting equation of standard curve with appropriate software interface. The software will calculate the concentration of samples after entering the OD Value of samples.

Figure: Typical Standard Curve: These standard curves are for demonstration only. A standard curve must be run with each assay..



X. RELATED PRODUCTS:

- 3-Nitrotyrosine Antibody (39B6) (Cat. No. K5412-100)
- Nitrotyrosine Antibody (Cat. No. 5416-50)
- Nitrotyrosine Antibody (7A5) (Cat. No. 5413-50)
- Nitrotyrosine Antibody (HM11) (Cat. No. 5414-50)
- Nitrotyrosine Antibody (NOY-7A5) (Cat. No. 5415-50)

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