

**User's Manual****Monkey Neopterin (Neopterin)ELISA Kit****REF****DEIA1640-M**

96T

RUO

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.

Creative Diagnostics **Address:** 45-1 Ramsey Road, Shirley, NY 11967, USA **Tel:** 1-631-624-4882 (USA) 44-161-818-6441 (Europe)  **Fax:** 1-631-938-8221 **Email:** info@creative-diagnostics.com  **Web:** www.creative-diagnostics.com

PRODUCT INFORMATION

Intended Use

In vitro quantitative determination of NPT concentrations in serum, plasma, cell culture supernatant and other biological samples.

Principles of Testing

This kit was based on Competitive-ELISA detection method. The microtiter plate provided in this kit has been pre-coated with NPT. During the reaction, NPT in the sample or standard competes with a fixed amount of NPT on the solid phase supporter for sites on the Biotinylated Detection Antibody specific to NPT. 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 TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of NPT in the samples is then determined by comparing the OD of the samples to the standard curve. The concentration of the target substance was inversely proportional to the OD450 value.

Reagents And Materials Provided

1. ELISA Microplate: (Dismountable) 8×12, 2-8°C/-20°C
2. Lyophilized Standard: 2 vial, 2-8°C/-20°C
3. Biotin-labeled Antibody (Concentrated, 100×): 1 vial, 120 µl, 2-8°C (Avoid Direct Light)
4. HRP-Streptavidin Conjugate (SABC, 100×): 120 µl, 2-8°C (Avoid Direct Light)
5. TMB Substrate: 10 ml, 2-8°C (Avoid Direct Light)
6. Sample Dilution Buffer: 20 ml, 2-8°C
7. Antibody Dilution Buffer: 10 ml 2-8°C
8. SABC Dilution Buffer: 10 ml, 2-8°C
9. Stop Solution: 10 ml, 2-8°C
10. Wash Buffer (25×): 30 ml, 2-8°C
11. Plate Sealer: 5 pieces
12. Product Description: 1 copy

Note: The liquid reagent bottle contains slightly more reagent than indicated on the label. Please use pipette accurately measure and do proportional dilution.

Materials Required But Not Supplied

1. Microplate reader (wavelength: 450 nm)
2. 37°C incubator (CO2 incubator for cell culture is not recommended.)
3. Automated plate washer or multi-channel pipette/5 ml pipettor (for manual washing purpose)

4. Precision single (0.5-10 μ L, 5-50 μ L, 20-200 μ L, 200-1000 μ L) and multi-channel pipette with disposable tips (calibration is required before use.)
5. Sterile tubes and Eppendorf tubes with disposable tips
6. Absorbent paper and loading slot
7. Deionized or distilled water

Storage

2-8°C/-20°C

Specimen Collection And Preparation

1. Serum

Place whole blood sample at room temperature for 2 hours or at 2-8°C overnight. Centrifuge for 20min at 1000xg and collect the supernatant to detect immediately. Or you can aliquot the supernatant and store it at -20°C or -80°C for future's assay.

2. Plasma

EDTA-Na2/K2 is recommended as the anticoagulant. Centrifuge samples for 15 minutes at 1000 $\times g$ 2-8°C within 30 minutes after collection. Collect the supernatant to detect immediately. Or you can aliquot the supernatant and store it at -20°C or -80°C for future's assay. For other anticoagulant types and uses, please refer to the sample preparation guideline.

3. Tissue Sample

Generally tissue samples are required to be made into homogenization. Protocol is as below:

3. 1. Place the target tissue on the ice. Remove residual blood by washing tissue with pre-cooling PBS buffer (0.01 M, pH = 7.4). Then weigh for usage.
3. 2. Use lysate to grind tissue homogenates on the ice. The adding volume of lysate depends on the weight of the tissue. Usually, 9 mL PBS would be appropriate to 1 gram tissue pieces. Some protease inhibitors are recommended to add into the PBS (e.g. 1 mM PMSF).
3. Do further process using ultrasonic disruption or freeze-thaw cycles (Ice bath for cooling is required during ultrasonic disruption; Freeze-thaw cycles can be repeated twice.) to get the homogenates.
3. 4. Homogenates are then centrifuged for 5 minutes at 5000 $\times g$. Collect the supernatant to detect immediately. Or you can aliquot the supernatant and store it at -20°C or -80°C for future's assay.
3. 5. Determine total protein concentration by BCA kit for further data analysis. Usually, total protein concentration for Elisa assay should be within 1-3mg/ml. Some tissue samples such as liver, kidney, pancreas which containing a higher endogenous peroxidase concentration may react with TMB substrate causing false positivity. In that case, try to use 1% H2O2 for 15min inactivation and perform the assay again.

Notes: PBS buffer or the mild RIPA lysis can be used as lysates. While using RIPA lysis, make the PH=7.3. Avoid using any reagents containing NP-40 lysis buffer, Triton X-100 surfactant, or DTT due to their severe inhibition for kits' working. We recommend using 50 mM Tris + 0.9% NaCL+0.1% SDS, PH7.3. You can prepare by yourself or contact us for purchasing.

4. Cell Culture Supernatant

 Tel: 1-631-624-4882 (USA)

Tel: 44-161-818-6441 (Europe)

 Fax: 1-631-938-8221

 Email: info@creative-diagnostics.com

Collect the supernatant: Centrifuge at 2500 rpm at 2-8°C for 5 minutes, then collect clarified cell culture supernatant to detect immediately. Or you can aliquot the supernatant and store it at -80°C for future's assay.

5. Cell Lysate

5. 1. Suspension Cell Lysate: Centrifuge at 2500 rpm at 2-8°C for 5 minutes and collect cells. Then add pre-cooling PBS into collected cell and mix gently. Recollect cell by repeating centrifugation. Add 0.5-1ml cell lysate and appropriate protease inhibitor (e.g. PMSF, working concentration: 1mmol/L). Lyse the cell on ice for 30min-1h or disrupt the cell by ultrasonic disruption.
5. 2. Adherent Cell Lysate: Absorb supernatant and add pre-cooling PBS to wash three times. Add 0.5-1ml cell lysate and appropriate protease inhibitor (e.g. PMSF, working concentration: 1mmol/L). Scrape the adherent cell with cell scraper. Lyse the cell suspension added in the centrifuge tube on ice for 30min-1h or disrupt the cell by ultrasonic disruption.
5. 3. During lysate process, use the tip for pipetting or intermittently shake the centrifugal tube to completely lyse the protein. Mucilaginous product is DNA which can be disrupted by ultrasonic cell disruptor on ice. (3~5 mm probe, 150-300 W, 3~5 s/time, 30s intervals for 1~2s working).
5. 4. At the end of lysate or ultrasonic disruption, centrifuge at 10000rpm at 2-8°C for 10 minutes. Then, the supernatant is added into EP tube to detect immediately. Or you can aliquot the supernatant and store it at -80°C for future's assay.

Notes: Read notes in tissue sample.

6. Other Biological Sample

Centrifuge samples for 15 minutes at 1000 ×g at 2-8°C. Collect the supernatant to detect immediately. Or you can aliquot the supernatant and store it at -80°C for future's assay.

Notes for Samples

1. Blood collection tubes should be disposable and non-endotoxin. Avoid to use hemolyzed and lipemia samples.
2. The best sample storage condition: less than 5 days at 2-8°C; within 6 months at -20°C; within 2 years at -80°C. Stored in liquid nitrogen for a longer storage. When melting frozen samples, rapid water bath at 15-25°C can decrease the effect of ice crystal (0°C) on the sample. After melting, centrifuge to remove the precipitate, and then mix well.
3. The detection range of this kit is not equivalent to the concentration of analyze in the sample. For analyses with higher or lower concentration, please properly dilute or concentrate the sample.
4. Pretest is recommended for special samples without reference data to validate the validity.
5. Recombinant protein may not match with the capture or detection antibody in the kit, resulting in the undetectable assay.

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

If other dilution ratio for your sample model is required, please refer to the universal dilution ratio below. (The ratio is suitable for single-well assay. For duplicate assay, please follow the calculation: volume of sample and diluent x number of duplicate well)

For 2 fold dilution (1/2): One step dilution. Add 60 µl sample into 60 µl sample diluent and mix gently.

For 5 fold dilution (1/5): One step dilution. Add 24 µl sample into 96 µl sample diluent and mix gently.

For 10 fold dilution (1/10): One step dilution. Add 12 µl sample into 108 µl sample diluent and mix gently.

For 20 fold dilution (1/20): One step dilution. Add 6 μ l sample into 114 μ l sample diluent and mix gently.

For 50 fold dilution (1/50): One step dilution. Add 3 μ l sample and 47 μ l normal saline (0.9% NaCl) into 100 μ l sample diluent and mix gently.

For 100 fold dilution (1/100): One step dilution. Add 3 μ l sample and 177 μ l normal saline into 120 μ l sample diluent and mix gently.

For 1000 fold dilution (1/1000): Two step dilution. Create a 50-fold dilution first (normal saline is used throughout the dilution). Then, create a 20-fold dilution and mix gently.

For 10000 fold dilution (1/10000): Two step dilution. Create a 100-fold dilution first (normal saline is used throughout the dilution). Then, create the same dilution again and mix gently.

For 100000 fold dilution (1/100000): Three step dilution. Create a 50-fold dilution and 20-fold dilution respectively (normal saline is used in the first two steps.) Finally, create a 100-fold dilution and mix gently.

Notes 1: The volume in each dilution is not less than 3 μ l. Dilution factor should be within 100 fold. Mixing during dilution is required to avoid foaming.

Reagent Preparation

Take the Elisa kit from the fridge around 20 minutes earlier and equilibrate to room temperature(18-25°C). For repeated assays, please just take the strips and standards required for the current assay, store the rest materials according to the relevant condition.

1. Wash Buffer

Dilute 30 ml concentrated wash buffer to 750 ml wash buffer with deionized or distilled water and mix well. (The recommended resistivity of ultrapure water is 18 M Ω .) Alternatively, take appropriate amount of concentrated wash buffer according to the assay requirement, then create a 25-fold dilution and mix well. Store the rest solution at 2-8°C.

Crystals formed in the concentrated wash buffer can be heated by water bath at 40°C till complete dissolution. (Heating temperature should be below 50°C.) Mix well for the next step. It's better to use up the prepared wash buffer in one day. Store the rest buffer at 2-8°C within 48h.

2. Standards

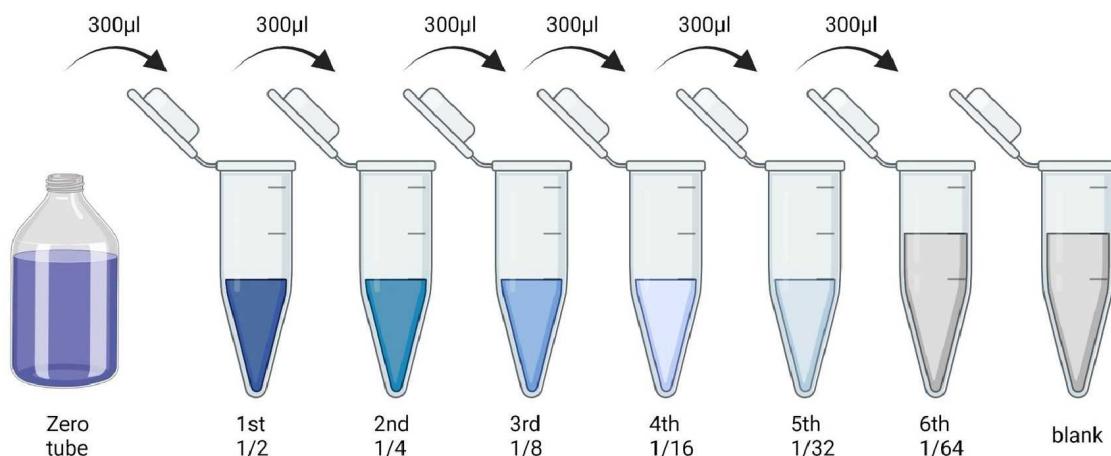
2.1. Centrifuge standards tube for 1 min at 10000 xg. Label it as Zero tube. **Note:** If the standard tube concentration higher than the range of the kit,please dilute it and labeled as zero tube.

2.2. Add 1 ml sample dilution buffer into the standard tube. Tighten the tube cap and Let it stand for 2 min at room temperature. Invert the tube several times to mix gently. (Or you can mix it using a low speed vortex mixer for 3-5 seconds.)

2.3. Centrifuge the tubes for 1min at 1000 xg, making the liquid towards the bottom of tube and removing possible bubbles.

2.4. Standard dilution: Label 7 EP tubes with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank respectively. Add 0.3 ml of the sample dilution buffer into each tube. Add 0.3 ml solution from zero tube into 1/2 tube and mix them thoroughly. Transfer 0.3 ml from 1/2 tube into 1/4 tube and mix them thoroughly.

Transfer 0.3 ml from 1/4 tube into 1/8 tube and mix them thoroughly, so on till 1/64 tube. Now blank tube only contain 0.3 ml sample dilution buffer.



Notes: It is best to use Standard Solutions within 2 hours.

3. Preparation of Biotin-labeled Antibody Working Solution

The working solution should be prepared within 30 min before the assay and can't be stored for a long time.

3.1. Calculate required total volume of the working solution: $100 \mu\text{l}/\text{well} \times \text{quantity of wells}$. (It's better to prepare additional $100 \mu\text{l} - 200 \mu\text{l}$.)

3.2. Centrifuge for 1min at 1000 xg in low speed and bring down the concentrated biotin-labeled antibody to the bottom of tube.

3.3. Dilute the biotinylated detection antibody with antibody dilution buffer at 1:99 and mix them thoroughly. (e.g. Add 10 µl concentrated biotin-labeled antibody into 990 µl antibody dilution buffer.)

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

The working solution should be prepared within 30 min before the assay and can't be stored for a long time.

4.1. Calculate required total volume of the working solution: $100 \mu\text{l}/\text{well} \times \text{quantity of wells}$. (It's better to prepare additional $100 \mu\text{l} - 200 \mu\text{l}$.)

4.2. Centrifuge for 1min at 1000 xg in low speed and bring down the concentrated SABC to the bottom of tube.

4.3. Dilute the concentrated SABC with SABC dilution buffer at 1:99 and mix them thoroughly. (e.g. Add 10 µl concentrated SABC into 990 µl SABC dilution buffer.)

Assay Procedure

When diluting samples and reagents, they must be mixed completely. It's recommended to plot a standard curve for each test.

1. Set standard, pilot samples, control (blank) wells on the pre-coated plate respectively, and then, records their positions. It's recommended to measure each standard and sample in duplicate to decrease experimental errors. Wash plate 2 times before adding standard, sample and control (blank) wells!
2. Standards and samples loading: Aliquot 50 µl of zero tube, 1st tube, 2nd tube, 3rd tube, 4th tube into each standard well. Also add 50 µl sample dilution buffer into the control (blank) well. Then, add 50 µl pilot samples into each sample well. Immediately add 50 µl Biotin-labeled Antibody Working Solution into each

well, gently tap the plate for 1min to ensure thorough mixing then static incubate for 45 minutes at 37° C. (Please keep tips or pipettors for adding Biotin-labeled Antibody away from the liquid level.)

3. Wash three times: Remove the cover, then absorb the liquid in the plate or tap the plate on a clean absorbent paper two or three times. Add 350 μ l wash buffer into each well and immerse for 1min. Discard the liquid in the well and tap on the absorbent paper again. Repeat the washing step three times.
4. HRP-Streptavidin Conjugate (SABC): Add 100 μ l SABC working solution into each well. Seal the plate and static incubate for 30 minutes at 37°C. (Put the whole bottle of TMB into the 37°C incubator to equilibrate)
5. Wash five times: Remove the cover, and then wash the plate with wash buffer five times. Read washing method in step 3.
6. TMB Substrate: Add 90 μ l TMB Substrate into each well, seal the plate and static incubate at 37°C in dark within 10-20 minutes. Run the microplate reader and preheat for 15min.

(Notes: Please do not use the reagent reservoirs used by HRP couplings. 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. Weaker or stronger color intensity is unacceptable.

7. Stop: Keep the liquid in the well after staining. Add 50ul stop solution into each well. The color will turn yellow immediately. The order for adding stop solution and TMB substrate solution is the same.
8. OD Measurement: Read the O. D. absorbance at 450nm in a microplate reader immediately. (If your microplate reader has a choice of correction wavelength, set it to 570nm or 630nm. Correct the read value to the OD450 value minus the OD570 or OD630 value. In this way, the OD value of non-chromogenic substances can be corrected and removed, thus obtaining more accurate results. If the microplate reader does not have a 570 nm or 630 nm wavelength, the original OD450 value can be used.)

Assay Procedure Summary

Step 1: Wash plate 2 times before adding Standard, Sample and Control (blank) wells!

Step 2: Add 50 μ l Standard or Sample into each well. Immediately add 50 μ l Biotin-labeled Antibody into each well, gently tap the plate for 1min to ensure thorough mixing then static incubate for 45 minutes at 37° C.

Washing: Wash the plate three times and immerse for 1min each time.

Step 3: Add 100 μ l SABC working solution into each well, seal the plate and static incubate for 30 minutes at 37° C.

Washing: Wash the plate five times and immerse for 1min each time.

Step 4: Add 90 μ l TMB substrate solution, seal the plate and static incubate for 10-20 minutes at 37°C. (Accurate TMB visualization control is required.)

Step 5: Add 50 μ l stop solution. Read at 450 nm immediately and calculate.

Calculation

1. Calculate the mean OD450 value (using the original OD450 value or the corrected OD450 value) of the duplicate readings for each standard, control, and sample. Then, obtain the value of calculation by subtracting the OD450 blank.
2. Create a four parameter logistic curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis. (Remove the OD450 blank during plotting.) Alternatively, you can

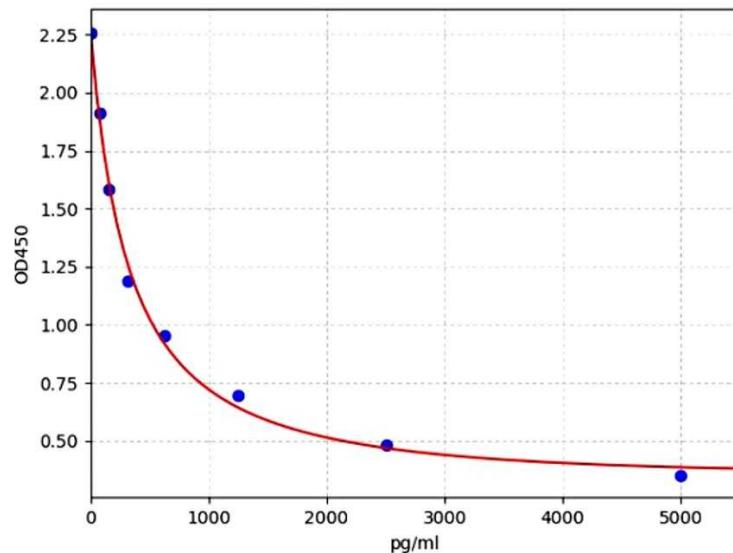
use the curve fitting software offered by the microplate reader.

- Calculate the sample concentration by substituting OD450 value into the standard curve. Diluted samples should be multiplied by the relevant dilution ratio.

Typical Standard Curve

The following assay data are provided for reference, since experimental environment and operation are different. The establishment of standard curve depends on your own assay.

STD.(pg/ml)	OD-1	OD-2	Average
0	2.225	2.289	2.257
78.125	1.886	1.94	1.913
156.25	1.56	1.606	1.583
312.5	1.171	1.205	1.188
625	0.94	0.968	0.954
1250	0.687	0.707	0.697
2500	0.473	0.487	0.48
5000	0.346	0.356	0.351



Precision

Intra-assay Precision: samples with low, medium and high concentration are tested 20 times on same plate.

Inter-assay Precision: samples with low, medium and high concentration are tested 20 times on three different plates.

Item	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/ml)	154	594.9	2457	158	616.9	2455
Standard deviation	8.16	30.82	125.8	8.04	31.03	130.36
CV(%)	5.3	5.18	5.12	5.09	5.03	5.31

Detection Range

78.125-5000 pg/ml

Sensitivity

46.875 pg/ml

Specificity

Specifically recognize NPT, no obvious cross reaction with other analogues

Linearity

Dilute the sample with a certain amount of NPT at 1:2, 1:4 and 1:8 to get the recovery range.

Matrix	1:2	1:4	1:8
Serum(n=10)	86-105%	82-99%	80-92%
EDTA Plasma(n=10)	85-103%	84-97%	83-98%
Heparin Plasma(n=10)	95-105%	86-101%	82-100%

Recovery

Add a certain amount of NPT into the sample. Calculate the recovery by comparing the measured value with the expected amount of NPT in the sample.

Matrix	Recovery Range (%)	Average (%)
Serum(n=10)	91-101	95
EDTA Plasma(n=10)	85-96	92
Heparin Plasma(n=10)	88-105	96

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. Please do not mix the reagents in different kits of our company. Do not mix reagents from other manufacturers.
10. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.