Manuals version 5.9

Cat No.ENMK06855

Rat TLR-3, TLR-4, TLR-5, TLR-6 Multiplex ELISA Kit

Analytes	Detection range	Sensitivity	Assay Length
TLR-3	0.078-5 ng/mL	0.026 ng/mL	4 hour
TLR-4	0.078-5 ng/mL	0.026 ng/mL	4 hour
TLR-5	0.078-5 ng/mL	0.026 ng/mL	4 hour
TLR-6	0.078-5 ng/mL	0.026 ng/mL	4 hour
Analytes	**Intra assay Precision**	**Inter assay Precision**	**Recovery Range**
TLR-3	CV ≤ 5%	CV ≤ 15%	81-113%
TLR-4	CV ≤ 6%	CV ≤ 9%	80-95%
TLR-5	CV ≤ 7%	CV ≤ 13%	82-103%
TLR-6	CV ≤ 5%	CV ≤ 9%	79-115%

Specifications	Details
Sample Types and Volume Required Per Well	Serum (100 μL), Plasma (100 μL), Cell Culture Supernatant (100 μL), Urine (100 μL), etc.
Storage	-20 ℃; short-term storage at 2-8 ℃ is acceptable for up to 2 weeks
Purpose	Intended for the in vitro quantitative determination of TLR-3, TLR-4, TLR-5, TLR-6 in samples
Specifications	96T
Production Date	Labeled on the outer packaging
Shelf Life	12 months when stored at -20 ℃

This kit is intended for the in vitro quantitative determination of four target analytes in serum, plasma, cell culture supernatants and tissue homogenate samples. For the detection of other special sample types, please contact our technical-support team. This kit is for research use only (RUO). Please read the instruction manual carefully before use and confirm that all kit components are complete.

This kit employs the "Double-Antibody-Sandwich" technique. The principle of Double-Antibody-Sandwich is based on the characteristics of a target analyte with at least two distinct epitopes, which can be simultaneously recognized by both the pre-coated capture antibody and the detection antibody. The process is as follows:

1. Coat the plate with capture antibodies, then wash to remove all unbound antibodies and impurities. The remaining sites on the plate are blocked with irrelevant proteins.
2. After blocking the remaining plate sites, add the samples containing the target analyte. The target analyte will be immobilized by the analyte-specific capture antibodies, forming an antigen-antibody complex. The wells are then washed to remove all unbound particles and impurities.
3. Biotin-labeled detection antibodies specific for each of the four target analytes is then added to the wells, forming an antibody-antigen-antibody complex. The plate is again washed to remove unbound antibodies and impurities.
4. Next, horseradish peroxidase-avidin-conjugate (HRP-avidin) is added to the wells and binds to the biotin-labeled antibodies. The quantity of reporter enzyme is positively correlated to the quantity of target

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analyte in the sample. The wells are then washed again to remove any residual impurities.

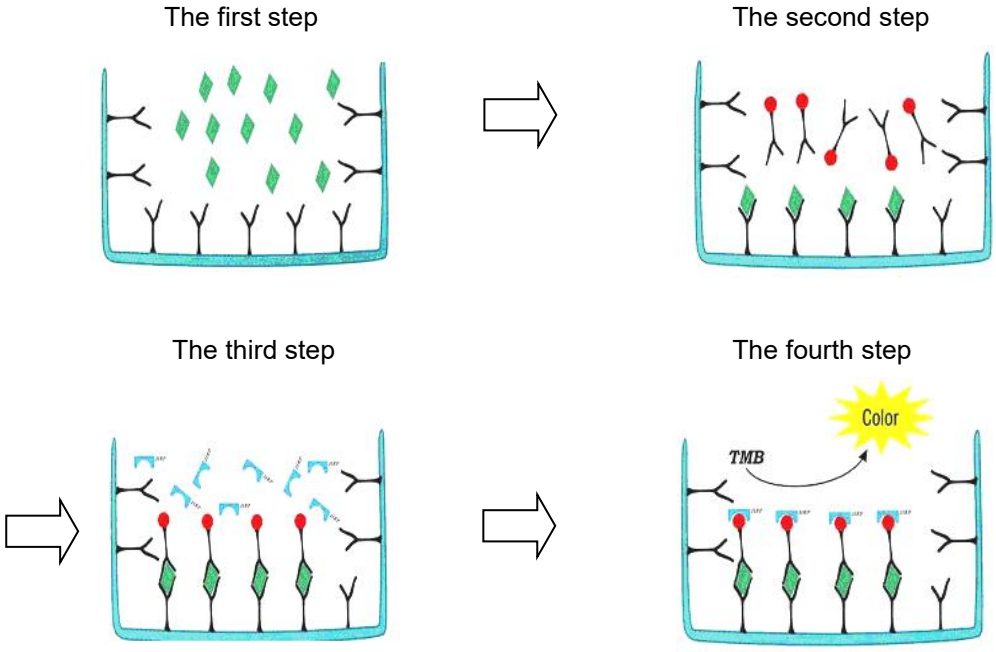
5. Finally, substrates for the horseradish peroxidase (HRP) reaction are added, and the sample concentrations can be calculated from the resulting color changes.

Note: Antibodies can be labeled with multiple biotin moieties, each of which can bind to HRP-avidin, resulting in multiple HRP complexes per antibody. This method exhibits higher sensitivity and stronger signal amplification compared to traditional direct HRP-labeled antibodies.

This kit adopts the Double-Antibody-Sandwich ELISA technique. The pre-coated microplate is separately coated with monoclonal antibodies against TLR-3, TLR-4, TLR-5 and TLR-6, and the detection reagent is a mixed biotin-conjugated polyclonal- antibody cocktail targeting the four analytes. Add samples and biotin-conjugated antibodies into the wells of the microplate, and wash with PBS or TBS washing buffer after each incubation step. Subsequently, avidin-peroxidase-conjugates are added to the wells. After thoroughly washing off unbound enzyme-conjugates with PBS or TBS, TMB substrate is added for color development.

TMB is oxidized by peroxidase to produce a blue-colored product, and the solution turns yellow upon addition of the stop solution (Color Reagent C). The color intensity is positively correlated with the concentration of target analytes in the sample.

General Schematic of the Double-Antibody Sandwich Principle:



Kit Components

Name	96 Tests	48 Tests	Storage
TLR-3(1-3), TLR-4(4-6), TLR-5(7-9), TLR-6(10-12) Pre-coated plate	8×12	8×6	2-8 ℃ or -20 ℃
TLR-3, TLR-4, TLR-5, TLR-6 Pre-Mixed Standards	2 vials	1 vial	2-8 ℃ or -20 ℃
Biotinylated TLR-3, TLR-4, TLR-5, TLR-6 Pre-Mixed antibody (1:100)	1 vial	1 vial	2-8 ℃ or -20 ℃
Enzyme-Conjugate(1:100)	1 vial	1 vial	2-8 ℃ or -20 ℃
TLR-3, TLR-4, TLR-5, TLR-6 Standard diluent	1 vial	1 vial	2-8 ℃ or -20 ℃
TLR-3, TLR-4, TLR-5, TLR-6 Antibody diluent	1 vial	1 vial	2-8 ℃ or -20 ℃
Enzyme diluent	1 vial	1 vial	2-8 ℃ or -20 ℃

Sample diluent	1 vial	1 vial	2-8 ℃ or -20 ℃
Washing buffer (1:25)	1 vial	1 vial	2-8 ℃ or -20 ℃
Color Reagent A	1 vial	1 vial	2-8 ℃ or -20 ℃
Color Reagent B	1 vial	1 vial	2-8 ℃ or -20 ℃
Color Reagent C	1 vial	1 vial	2-8 ℃ or -20 ℃
Manual	1 set	1 set	RT

Notes:

RT: Room Temperature

Standard: Lyophilized

Color Reagent A: Protect from light

Materials Required But Not Provided With Kit

1. Microplate reader (equipped with a 450 nm detection wavelength filter; optional 570 nm or 630 nm correction wavelength filters are recommended)
2. Microplate washer (adjustable injection volume to ensure 350 μL is dispensed into each well without overflow)
3. Clean benches, biological safety cabinets, fume hoods
4. High-precision single-channel pipette (ranges: 0.5-10 μL, 20-200 μL, 200-1000 μL)
5. High-precision multi-channel pipette (8-channel or 12-channel, range: 50-300 μL)
6. 37 ℃ incubator
7. Refrigerated centrifuge
8. Refrigerators (capable of maintaining temperatures at 4 ℃, -20 ℃ and -86 ℃)
9. Analytical balance
10. Scissors, tweezers, forceps, etc.
11. Plate mixer, low-frequency oscillator, etc.

Additional Materials Required

1. Centrifuge tubes (capacities: 1.5 mL, 5 mL, etc.)
2. Disposable pipette tips (ranges: 0.5-10 μL, 20-200 μL, 200-1000 μL)
3. Pure water or distilled water
4. Semi-log graph paper
5. Absorbent paper
6. EDTA, sodium citrate and heparin

Sample Collection Notes

1. Blood collection tubes should be both pyrogen- and endotoxin-free.
2. Hemolyzed or hyperlipidemic specimens are not recommended for use.
3. Samples should be clear and transparent; all particulates must be removed by centrifugation.
4. If collected samples are not used immediately, aliquot them into single-use portions and store frozen at -20 ℃ to -80 ℃. Avoid repeated freeze-thaw cycles.
5. Optimization of sample dilution is often necessary to ensure that sample concentrations fall within the linear range of the standard curve. Pre-experiments are recommended prior to testing the bulk samples to determine the optimal dilution ratio.
6. It is recommended to collect sufficient sample volumes for replicate testing, as unforeseen issues may lead to data loss.
7. Always wear protective clothing when collecting or handling samples (e.g., gloves, lab coat, respirator, etc.) and be aware of all potential risks associated with specimen handling.
8. Specimen processing should be conducted in a properly maintained biological safety cabinet.

Sample Preparation

Serum: Place the collected whole blood in a 4 ℃ refrigerator overnight. Then centrifuge at 1000-3000 rpm for 10 min. Collect the supernatant and either test immediately or store the samples at -20 ℃ or -80 ℃ for 1-3 months.

Plasma: Use EDTA, sodium citrate, or heparin as anticoagulants. Add the selected anticoagulant to the collected whole blood, mix well, and then separate the plasma. Centrifuge the plasma mixture at 1000-3000 rpm for 10 min. Collect the supernatant and either test immediately or store the samples at -20 ℃ or -80 ℃ for 1-3 months.

Tissue Homogenate: Take the tissue sections and rinse them with 0.01 M PBS. Add tissue protein extraction reagent at a ratio of 1 g tissue to 5-10 mL reagent, and mix thoroughly in an ice bath. After sufficient homogenization, centrifuge at 5000-10000 rpm for 10 min. Collect the supernatant for immediate testing, or store the samples at -20 ℃ or -80 ℃ for 1-3 months.

Cell Culture Supernatant: Centrifuge the collected cell culture supernatant at 1000-3000 rpm for 10 min. Collect the supernatant for immediate testing, or store the samples at -20 ℃ or -80 ℃ for 1-3 months.

Special Samples (Urine, Ascites, Cerebrospinal Fluid, etc.): Centrifuge the samples at 1000-3000 rpm for 10 min. Collect the supernatant for immediate testing, or store the samples at -20 ℃ or -80 ℃ for 1-3 months.

Note: It is recommended that users be familiar with the expected concentration range of their samples. We suggest consulting the scholarly literature for the concentration ranges of similar samples, and then adjusting the sample dilution ratio accordingly.

Note

1. The reconstituted standard cannot be stored after preparation; do not refreeze the standard once it has been reconstituted.
2. Due to shaking or inversion during transportation, centrifugation of the kit tubes or bottles may be necessary to collect all contents at the bottom. Tubes should be vortexed manually or centrifuged at 1000 rpm for 1 min.
3. Concentrated washing buffer may crystallize slightly. Heat the buffer in a water bath to facilitate dissolution during dilution. Crystals must be completely dissolved before the washing buffer is used.
4. The prepared standard is for single-use only; do not reuse any standard that has already been assayed. Use the second vial provided if the assay needs to be repeated.
5. Use only the reagents and components provided with this kit. Do not mix batches or lots from other orders of this kit or from different kit types.
6. Ensure all reagents are thoroughly mixed. For reagents added to the microplate, adequate mixing is critical for obtaining accurate assay results. A microplate shaker (set to the lowest frequency) is recommended. If a shaker is not available, manually shake the microplate gently in a circular motion for 1 min to ensure uniform mixing in all wells.
7. Bring the kit to room temperature before starting the assay.
8. It is recommended to test all standards in duplicate or triplicate.
9. Store unused microplate strips in the original foil bag at 2-8 ℃ if they will be used within a short period.
10. The chromogen reagent is light-sensitive; avoid exposure to light.
11. Do not use kits that have exceeded their expiration date.
12. When using dual-wavelength detection, set the wavelengths to 450 nm and 630 nm.
13. All samples, washing buffer, and wastes should be treated as biohazardous waste. Color Reagent C is 1 M sulfuric acid; handle this reagent with extreme care.
14. Sample addition must be performed using a pipette or similar calibrated instrument. Calibrate the instrument before the assay to avoid experimental errors. Add samples to the wells quickly, and control the total sample addition time to less than 5 minutes. A multichannel pipette is recommended to reduce

loading time.

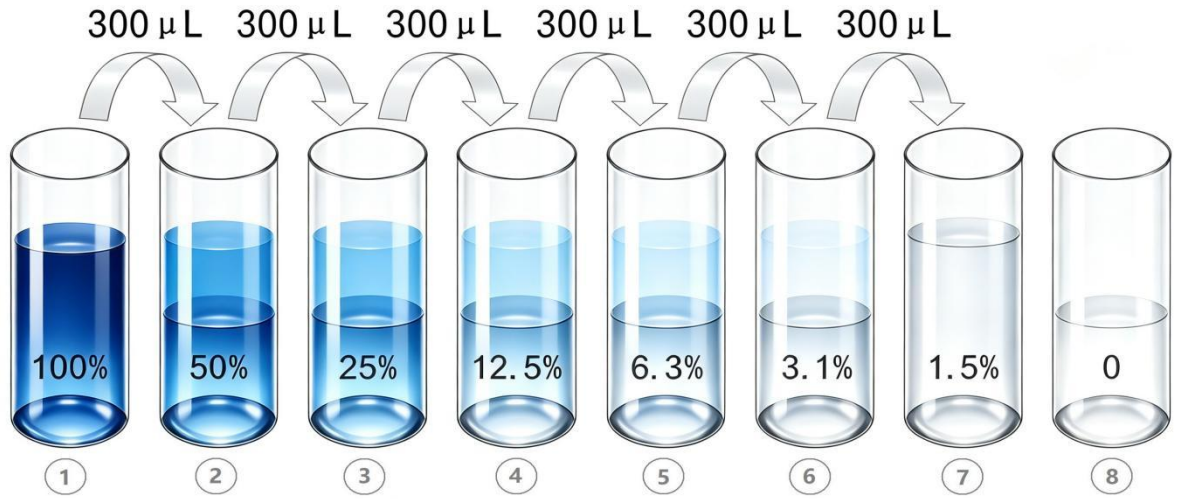
15. Do not reuse adhesive plate seals. Cut the seal to size if only part of the plate is used, and discard the seal after use.
16. A new standard curve must be generated for each independent assay run. If the detected concentration of a sample is too high (i.e., the OD value exceeds that of the highest-concentration standard well), dilute the sample by a specific factor and correct the final calculation accordingly.
17. Samples containing sodium azide (NaN_3) cannot be assayed, as NaN_3 inhibits the enzymatic activity of horseradish peroxidase (HRP).
18. When using a Microplate washer, ensure the buffer volume per well is slightly more than 350 μL, and check that the pipetting probe is not clogged. If washing manually, use absorbent paper to remove excess buffer, and ensure the paper has not been in contact with other reagents to prevent cross-contamination.
19. Read the OD values within 10 minutes after terminating the color reaction with Color Reagent C.
20. For samples tested in duplicate wells, use the mean OD value of the duplicate wells for calculations.
21. Hemolyzed samples may cause false positive results and are not suitable for use with this kit.
22. Maintain a relative humidity of approximately 60% during the assay.
23. Regularly verify the calibration of the incubator to ensure a stable incubation temperature of 37 ℃.
24. For the 48-well ELISA Kit, all component volumes are 50% of those provided in the 96-well kit.

Test preparation

1. Remove the ELISA kit from the refrigerator 20 min in advance, and start the assay once it has equilibrated to room temperature.
2. Dilute the concentrated washing buffer with double-distilled water at a ratio of 1:25. Store the unused portion back in the original kit box.
3. Standard Preparation: Add 1.0 mL of Standard Diluent to the lyophilized standard vial, and allow it to stand for 30 minutes. After the standard has completely dissolved, mix it gently by inverting the vial several times, and clearly label it as STD1. Note: Ensure that the lyophilized standard is completely dissolved and thoroughly mixed.
4. Procedure for standard sample dilution: Take 7 clean tubes and label them with their expected concentrations (STD2, STD3, STD4, STD5, STD6, STD7, STD8). Add 300 μL of Standard Diluent to each tube. Pipette 300 μL from the reconstituted STD1 into the tube labeled STD2 and mix well. Then pipette 300 μL from the STD2 tube into the STD3 tube and mix well. Repeat this procedure up to the STD7 tube. The Standard Diluent in the STD8 tube serves as the negative control.

Analytes	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	concentration
TLR-3	5	2.5	1.25	0.625	0.313	0.156	0.078	0	ng/mL
TLR-4	5	2.5	1.25	0.625	0.313	0.156	0.078	0	ng/mL
TLR-5	5	2.5	1.25	0.625	0.313	0.156	0.078	0	ng/mL
TLR-6	5	2.5	1.25	0.625	0.313	0.156	0.078	0	ng/mL

Note: The reconstituted standard solution (STD1: TLR-3 5 ng/mL, TLR-4 5 ng/mL, TLR-5 5 ng/mL, TLR-6 5 ng/mL) should be discarded after running the assay - it is not reusable.



Note: Reconstituted standard stock solution cannot be reused.

5. Biotinylated Antibody: Remove the appropriate volume of Biotinylated Antibody solution based on the number of wells to be assayed, and dilute it with Antibody Diluent at a 1:100 ratio. Mix the solution thoroughly after dilution. Prepare this working solution 30 min in advance, and do not reuse the diluted solution for subsequent assays.
6. Enzyme-Conjugate: Remove the appropriate volume of Enzyme-Conjugate solution based on the number of wells to be assayed, and dilute it with Enzyme Diluent at a 1:100 ratio. Mix the solution thoroughly after dilution. Prepare this working solution 30 min in advance, and do not reuse the diluted solution for subsequent assays.
7. Color Reagent: Prepare the Color Reagent working solution 30 min in advance by mixing Color Reagent A and Color Reagent B at a ratio of 9:1.

Washing method

1. Automatic Plate Washing: Use 350 μL of wash buffer per well, with an interval of 20-30 seconds between buffer dispensing and aspiration. Familiarize yourself with the instrument operation manual before use.
2. Manual Plate Washing: Add 350 μL of wash buffer to each well and allow to stand for 30 seconds. Shake the plate vigorously to remove as much liquid as possible, and pat the plate firmly on absorbent paper if necessary. During the washing process, pay close attention to the buffer addition steps to avoid cross-contamination and missing wells.

Steps

1. Remove the required number of microplate strips and allow them to equilibrate to room temperature. Return the unused strips and desiccant to the original sealed aluminum foil bag and store at 2-8 ℃.
2. Set aside blank wells (blank wells may be omitted if using dual-wavelength detection).
3. Add standards or samples to their corresponding wells (100 μL per well). Note that the 0 ng/mL standard well should be filled with 100 μL of Standard Diluent. Seal the plate with an adhesive plate seal and incubate at 37 ℃ for 90 min (protect from light).
4. Prepare the required volume of Biotinylated Antibody 30 min in advance.
5. Wash the ELISA plate 2 times with the provided washing buffer.
6. Add the prepared Biotinylated Antibody to each well (100 μL per well). Seal the plate with an adhesive plate seal and incubate at 37 ℃ for 60 min (protect from light).
7. Prepare the required volume of Enzyme-Conjugate 30 min in advance.
8. Wash the ELISA plate 3 times with the provided washing buffer.
9. Add the prepared Enzyme-Conjugate to all wells except the blank wells (100 μL per well). Seal the plate

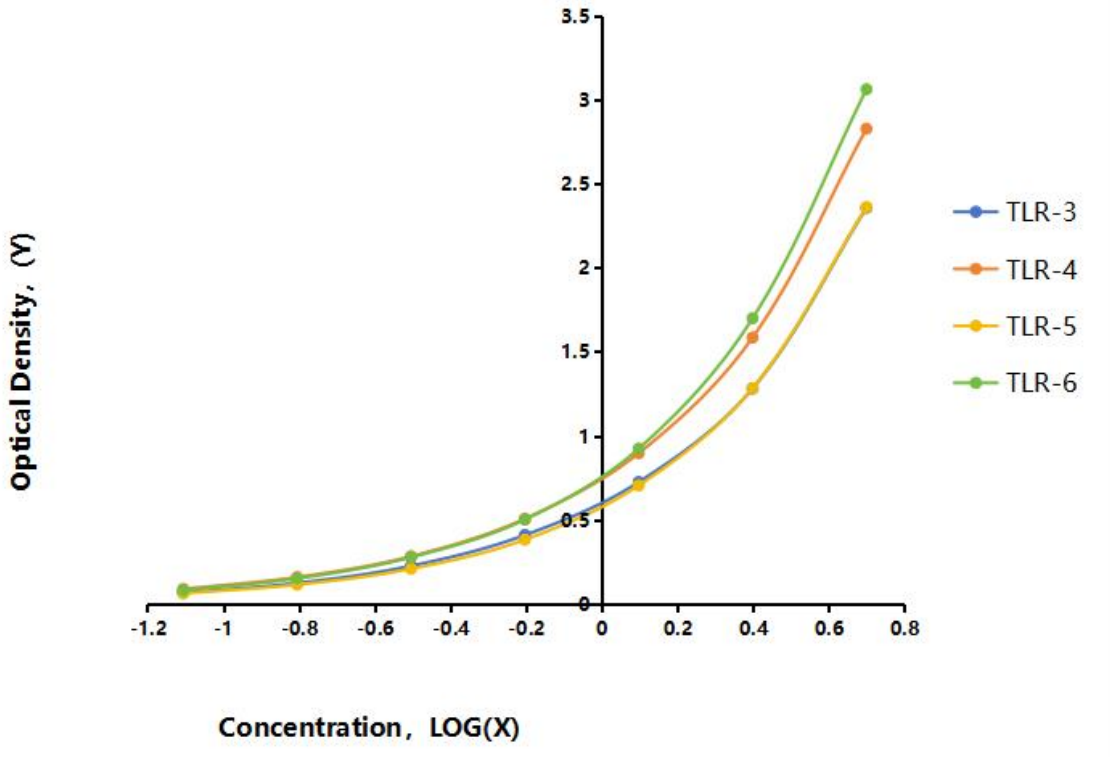
with an adhesive plate seal and incubate at 37 ℃ for 30 min (protect from light).

10. Wash the ELISA plate 5 times with the provided washing buffer.
11. Add 100 µL of the prepared TMB Substrate Solution to each well (including blank wells). Incubate protected from light at 37 ℃ for 10-30 min. Terminate the reaction when the highest-concentration standard wells show obvious color development and a clear color gradient is observed (the chromogenic reaction time should not exceed 30 min).
12. Add 100 µL of Color Reagent C to each well (including blank wells), mix thoroughly. Read the OD value at 450 nm (or 450 nm/630 nm for dual-wavelength detection) within 10 min.

Result determination

1. The OD values of each sample and standard should be corrected by subtracting the OD value of the blank well.
2. Draw the standard curve manually: Plot the standard curve using the standard concentrations as the X-axis and the corresponding OD values as the Y-axis. Connect the plotted points with a smooth curve. The concentration of samples can be calculated by substituting the sample OD values into the standard curve equation. It is recommended to use professional curve-fitting software (e.g., Curve Expert 1.3) for result analysis and calculation.
3. If the sample OD value is higher than that of the highest-concentration standard in the standard curve, the sample should be diluted (or further diluted) and reassayed. Multiply the measured concentration by the dilution factor when calculating the final concentration.
4. Standard curve can also be plotted with logarithmic-transformed concentrations, or plotted on a semi-logarithmic coordinate system.Note: This protocol is for reference only. The concentration calculation of test samples must be based on the standard curve generated during the same experiment.

Reference curve



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Note: Again, please note that this chart is for reference only, and that user sample concentrations should be based on the standard curve generated in the same assay run on the same microplate.

Summary of operating procedures

step	Summary of operating procedures
1	Prepare reagents, samples and standards.
2	Add the prepared samples and standards to their respective wells and seal the plate. Incubate at 37 ℃ for 90 minutes.
3	Wash the plate 2 times. Add the Biotinylated Antibody solution and seal the plate. Incubate at 37 ℃ for 60 minutes.
4	Wash the plate 3 times. Add the prepared Enzyme-Conjugate working solution and seal the plate. Incubate at 37 ℃ for 30 minutes.
5	Wash the plate 5 times. Add the TMB Substrate Solution and incubate at 37 ℃ in the dark for 10-30 minutes until a clear color gradient appears.
6	Add Color Reagent C to each well to terminate the reaction.
7	Use a microplate reader to measure the OD value at 450 nm within 10 minutes of adding Color Reagent C.
8	Calculate the concentration of the test samples.