Version 06-08/24

User's Manual

FFA (Free Fatty Acid) ELISA Kit

REF **DEIA-JY2421**

Σ **96T**

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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

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PRODUCT INFORMATION

Intended Use

For detection of Free Fatty Acid in test 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 FFA. During the reaction, FFA in the sample or standard competes with a fixed amount of FFA on the solid phase supporter for sites on the Biotinylated Detection Antibody specific to FFA. 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 450nm. The concentration of FFA 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. Lyophilized Standard: 2
3. Biotin-labeled Antibody (Lyophilized): 1
4. HRP-Streptavidin Conjugate (SABC, 100×): 120 µL
5. TMB Substrate: 10 mL
6. Purified water: 200 µL
7. Sample Dilution Buffer: 20 mL
8. Antibody Dilution Buffer: 10 mL
9. SABC Dilution Buffer: 10 mL
10. Stop Solution: 10 mL
11. Wash Buffer (25×): 30 mL
12. Plate Sealer: 5 pieces

Materials Required But Not Supplied

1. Microplate reader (wavelength: 450nm)
2. 37°C incubator (CO_2 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 µL, 5-50 µL, 20-200 µL, 200-1000 µL) 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 (for sealed box), please do not freeze! See kit label for expiry date.

Specimen Collection And Preparation

1. **Serum**

Place whole blood sample at room temperature for 2 hours or at 2-8°C overnight. Centrifuge for 20 min at 1000 ×g 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-Na_2/K_2 is recommended as the anticoagulant. Centrifuge samples for 15 minutes at 1000 ×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 normal saline (0.9% NaCl). Then weigh for usage.

3.2. Use normal saline (0.9% NaCl) to grind tissue homogenates on the ice. The adding volume of normal saline (0.9% NaCl) depends on the weight of the tissue. Usually, 9 mL normal saline (0.9% NaCl) would be appropriate to 1 gram tissue pieces. Some protease inhibitors are recommended to add into the PBS (e.g. 1mM PMSF).

3.3. 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 ×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 1-3 mg/ml.

Note: The use of other lysates is not recommended, which can cause precipitation and non-color development during TMB incubation.

4. **Cell Culture Supernatant**

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 precooling PBS into collected cell and mix gently. Recollect cell by repeating centrifugation. Add 0.5-1 mL cell lysate and appropriate protease inhibitor (e.g. PMSF, working concentration: 1 mmol/L). Lyse the

cell on ice for 30 min-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. (3~5mm probe, 150-300W, 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.

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.

Recommended Sample Dilution Ratio

Please refer to shipped instructions or contact us for samples, dilution as well background info.

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 µL sample into 114 µL sample diluent and mix gently.

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

For 100 fold dilution (1/100): One step dilution. Add 3 µL sample and 177 µL normal saline into 120 µL 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: The volume in each dilution is not less than 3 μL. 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 18MΩ.) 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.

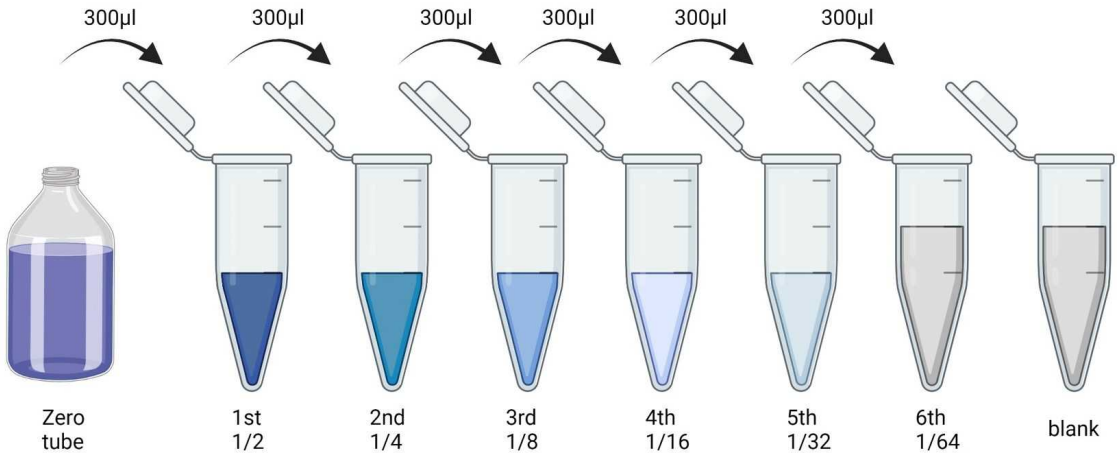
2. **Standards**

2.1. Centrifuge standards tube for 1min at 10000 xg. Label it 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. The standard concentration from zero tube to blank tube is 400 nmol/mL, 200 nmol/mL, 100 nmol/mL, 50 nmol/mL, 25 nmol/mL, 12.5 nmol/mL, 6.25 nmol/mL, 0 nmol/mL.



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. Dissolve: Centrifuge for 1min at 2000 ×g and bring down the concentrated biotin-labeled antibody to the bottom of tube. Add 70 μL purified water into tube and mix them thoroughly, after the biotin-labeled antibody is dissolved, please store it at 2-8°C.

   3.2. Calculate required total volume of the working solution: 50 μL/well × quantity of wells. (It's better to prepare additional 100 μL-200 μL.)

   3.3. Dilute the biotinylated detection antibody with antibody dilution buffer at 1/100 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 μL/well x quantity of wells. (It's better to prepare additional 100 μL-200 μL.)

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 1/100 and mix them thoroughly. (e.g. Add 10 μ L concentrated SABC into 990 μL SABC dilution buffer.)

Assay Procedure Summary

Step1: Wash plate 2 times before adding Standard, Sample and Control (blank) wells!

Step2: Add 50 μL Standard or Sample into each well. Immediately add 50ul 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.

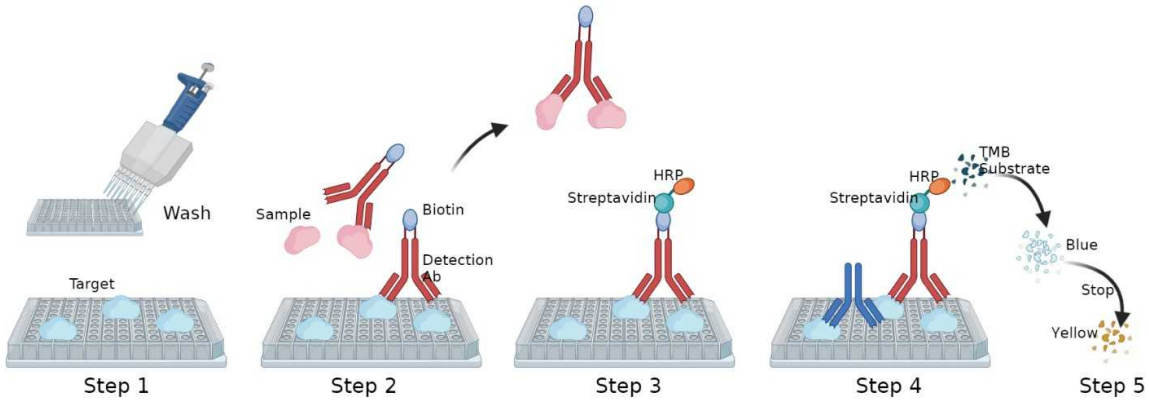
Washing: Wash the plate three times and immerse for 1min each time.

Step 3: Add 100 μL 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 μL 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 µL stop solution. Read at 450 nm immediately and calculate.



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.)
2. 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 µL 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 µL 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 µL 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 15 min.

(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 450 nm in a microplate reader immediately. (If your microplate reader has a choice of correction wavelength, set it to 570 nm or 630 nm. 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.)

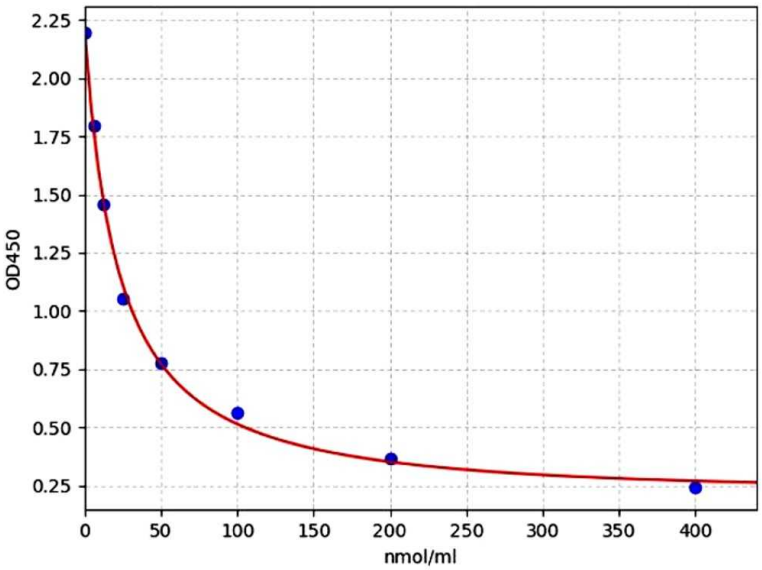
Calculation

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.
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. Alternatively, you can use the curve fitting software offered by the microplate reader.
3. 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.(nmol/ml)	OD-1	OD-2	Average
0	2.163	2.225	2.194
6.25	1.773	1.825	1.799
12.5	1.437	1.479	1.458
25	1.038	1.068	1.053
50	0.766	0.788	0.777
100	0.554	0.57	0.562
200	0.359	0.369	0.364
400	0.239	0.245	0.242



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		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (nmol/ml)	13.2	48.96	204.5	12.86	50.35	208.9
Standard deviation	0.7	2.27	9.57	0.61	2.32	9.9
CV(%)	5.34	4.63	4.68	4.71	4.61	4.74

Detection Range

6.25-400 nmol/mL

Detection Limit

3.75 nmol/mL

Specificity

Specifically recognize FFA, no obvious cross reaction with other analogues.

Linearity

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

Matrix	1:2	1:4	1:8
Serum(n=10)	87-98%	83-99%	83-99%
EDTA Plasma(n=10)	85-104%	83-100%	80-100%
Heparin Plasma(n=10)	85-100%	85-101%	84-95%

Recovery

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