Technical Manual

5-HETE ELISA Kit

- Catalogue Code: UNES00062
- Competitive ELISA Kit
- Research Use Only

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1. Description and Principle

This ELISA kit applies to the in vitro quantitative determination of 5-Hydroxyeicosatetraenoic Acid concentrations in serum, plasma and other biological fluids.

How Do Our ELISA Kit Assays Work?

This ELISA kit uses the competitive ELISA principle. The ELISA plate provided in this kit has been pre-coated with 5-Hydroxyeicosatetraenoic Acid. During the reaction, 5-Hydroxyeicosatetraenoic Acid in the sample or standard competes with a fixed amount of 5-Hydroxyeicosatetraenoic Acid on the solid phase support for sites on the Biotinylated Detection Ab specific to 5-Hydroxyeicosatetraenoic Acid. Excess conjugate and unbound sample or standard are washed from the plate, and Avidin-HRP is added to each well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of stop solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of 5-Hydroxyeicosatetraenoic Acid in the samples is determined by comparing the OD of the samples to the standard curve.

2. Key features and Sample Types

Sensitivity: 0.13 ng/mL

Detection Range: 0.16 - 10 ng/mL

ELISA Type: Competitive

Specificity: This kit recognizes 5-Hydroxyeicosatetraenoic Acid in samples. No significant cross-reactivity or interference between 5-Hydroxyeicosatetraenoic Acid and analogues was observed.

SUMMARY

1. Add 50μL standard or sample to each well. Immediately add 50μL Biotinylated Detection Ab to each well. Incubate for 45 min at 37℃.

2. Aspirate and wash 3 times.

3. Add 100μL HRP Conjugate to each well. Incubate for 30 min at 37℃.

4. Aspirate and wash 5 times.

5. Add 90μL Substrate Reagent. Incubate 15 min at 37℃.

6. Add 50μL Stop Solution. Read at 450nm immediately.

7. Calculation of results.

3. Kit Contents

Product	Size	Cat. Code
5-HETE ELISA Kit	24/96 assays	UNES00062

Each kit contains reagents for 24/96 assays in a 24/96 well plate including:

Item	24T	96T	Storage
Micro ELISA Plate (Dismountable)	8 wells x 3 strips	8 wells x 12 strips	-20°C, 6 months
Reference Standard	1 vial	2 vials	
Concentrated Biotinylated Detection Ab (100×)	1 vial, 60 µL	1 vial, 120 µL	
Concentrated HRP Conjugate (100×)	1 vial, 60 µL	1 vial, 120 µL	-20°C (shading light), 6 months
Reference Standard & Sample Diluent	1 vial, 20 mL	1 vial, 20 mL	4°C, 6 months
Biotinylated Detection Ab Diluent	1 vial, 14 mL	1 vial, 14 mL	
HRP Conjugate Diluent	1 vial, 14 mL	1 vial, 14 mL	
Concentrated Wash Buffer (25×)	1 vial, 30 mL	1 vial, 30 mL	
Substrate Reagent	1 vial, 10 mL	1 vial, 10 mL	4°C (shading light)
Stop Solution	1 vial, 10 mL	1 vial, 10 mL	4°C
Plate Sealer	5 pieces	5 pieces	
Product Description	1 copy	1 copy	

Additional Materials required

1. Microplate reader with 450 nm wavelength filter
2. High-precision transfer pipette, EP tubes and disposable pipette tips
3. Incubator capable of maintaining 37°C
4. Deionized or distilled water
5. Absorbent paper
6. Loading slot for Wash Buffer

4. Shipping and Storage

An unopened kit can be stored at 4°C for 1 month. If the kit is not used within 1 month, store the items separately according to the vial labels.

5. Sample Preparation

Serum: Allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 15 min at 1000×g at 2~8°C. Collect the supernatant to carry out the assay. Blood collection tubes should be disposable and be endotoxin-free.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 min at 1000×g at 2~8°C within 30 min of collection. Collect the supernatant to carry out the assay. Hemolysed samples are not suitable for ELISA assay.

Cell lysates: For adherent cells, gently wash the cells with pre-cooled PBS and dissociate the cells using trypsin. Collect the cell suspension in a tube and centrifuge for 5 min at 1000×g. Discard the medium and wash the cells 3 times with precooled PBS. For each 1×10^6 cells, add 150-250µL of pre-cooled PBS to keep the cells suspended. 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 at 4°C for 20 mins at 2000-3000 rpm to pellet debris and remove clear supernatant to clean microcentrifuge tube for ELISA or storage.

Tissue homogenates: It is recommended to get detailed references from the literature before analyzing different tissue types. For general information, hemolysed blood may affect the results, so the tissues should be minced into small pieces and rinsed in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then homogenized in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogenizer on ice. To further break down 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 min at 5000×g to get the supernatant.

Cell culture supernatant or other biological fluids: Centrifuge samples for 20 min at 1000×g at 2~ 8°C. Collect the supernatant to carry out the assay.

Notes:

1. Samples should be assayed within 7 days when stored at 4°C, otherwise samples must be divided up and stored at -20°C (≤1 month) or -80°C (≤3 months). Avoid repeated freeze-thaw cycles.

2. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

3. If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.

4. If a lysis buffer is used to prepare tissue homogenates or cell culture supernatant, there is a possibility of causing a deviation due to the introduced chemical substance.

5. Some recombinant protein may not be detected due to a mismatching with the coated antibody or detection antibody.

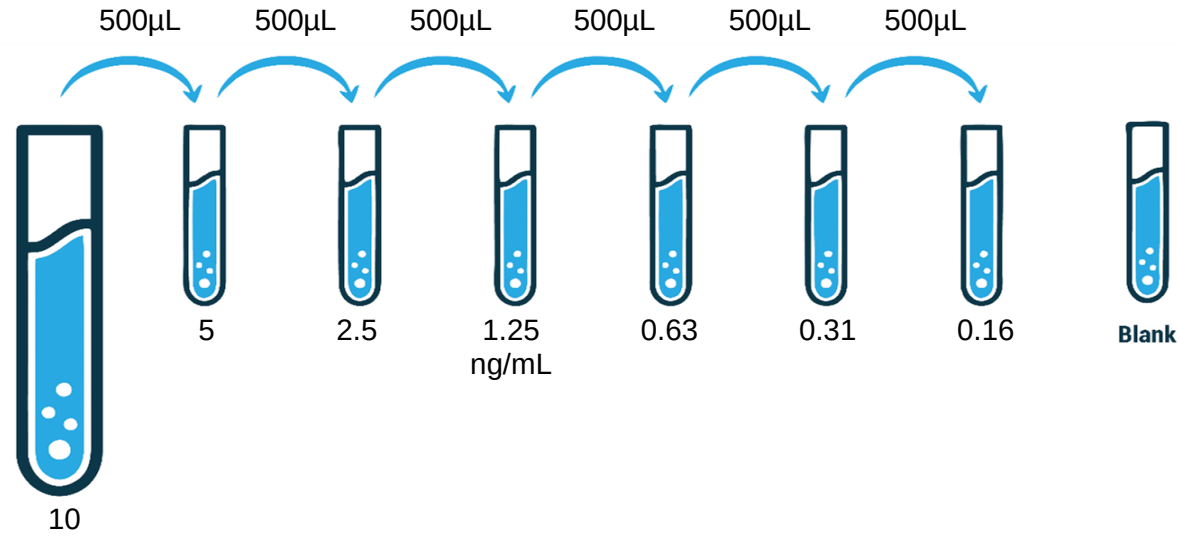
6. Protocol

1. Bring all reagents to room temperature: (18~25°C) before use. Follow the Microplate reader manual for set-up and preheat it for 15 min before OD measurement.

2. Wash Buffer: Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: if crystals have formed in the concentrate, warm it in a 40°C water bath and mix it gently until the crystals have completely dissolved.

3. Standard working solution: Centrifuge the standard at 10,000×g for 1 min. Add 1.0 mL of Reference Standard & Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 10 ng/mL. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0 ng/mL. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube.

Dilution method: Take 7 EP tubes, add 500µL of Reference Standard & Sample Diluent to each tube. Pipette 500µL of the 10 ng/mL working solution to the first tube and mix up to produce a 5 ng/mL working solution. Pipette 500µL of the solution from the former tube into the latter one according to these steps. The illustration below is for reference. Note: the last tube is regarded as a blank.



4. Biotinylated Detection Ab working solution: Calculate the required amount before the experiment (50µL/well), slightly more than calculated should be prepared. Centrifuge the stock tube before use, dilute the 100× Concentrated Biotinylated Detection Ab to 1×working solution with Biotinylated Detection Ab Diluent.

5. Concentrated HRP Conjugate working solution: Calculate the required amount before the experiment (100µL/well), slightly more than calculated should be prepared. Dilute the 100× Concentrated HRP Conjugate to 1× working solution with Concentrated HRP Conjugate Diluent.

7. Assay procedure

1. Set standard, test sample and control (zero) wells on the pre-coated plate and record their positions. It is recommended to measure each standard and sample in duplicate. Note: add all solutions to the bottom of the plate wells while avoiding contact with the well walls. Ensure solutions do not foam when adding to the wells.

2. Add 50µL of Standard, Blank or Sample to their respective wells. The blank well is added with Sample / Standard dilution buffer.

3. Immediately add 50 µL of Biotin-detection antibody working solution to each well.

4. Cover with a plate seal and gently tap the plate to ensure thorough mixing. Incubate for 45 minutes at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

5. Aspirate or decant the solution from the plate and add 350µL of wash buffer to each well and incubate for 1-2 minutes at room temperature. Aspirate the solution from each well and clap the plate on absorbent filter paper to dry. Repeat this process 3 times. Note: a microplate washer can be used in this step and other wash steps.

6. Add 100µL of HRP Conjugate working solution to each well and over with a plate seal. Incubate for 30 minutes at 37°C.

7. Repeat the aspiration/wash process 5 times according to step 5.

8. Add 90µL of the Substrate reagent to each well and cover with a new plate seal. Incubate for approximately 15 minutes at 37°C and protect from light. The reaction time can be shortened or extended according to the colour change, but not by more than 30 minutes. When apparent gradient appears in standard wells, terminate the reaction.

9. Stop: Add 50µL of Stop Solution to each well (wells will develop a yellow color immediately). Note: Adding the stop solution should be done in the same order as the substrate solution.

10. Determine the optical density (OD value) of each well immediately with a microplate reader set at 450 nm. In advance, preheat the instrument and set the testing parameters.

8. Data analysis

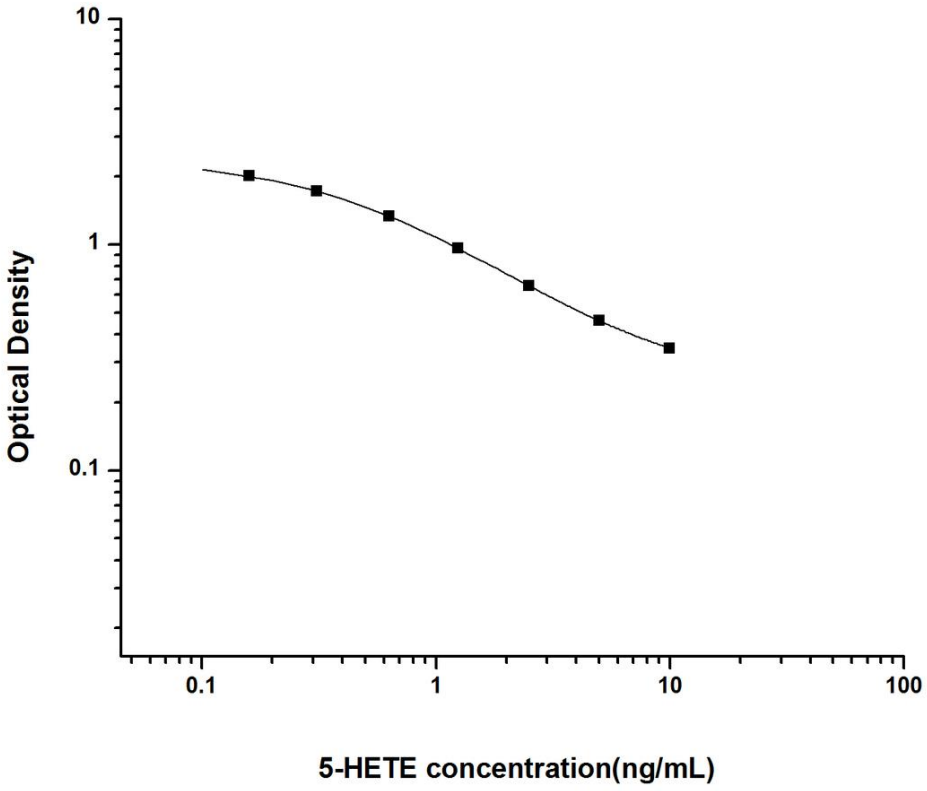
Average the duplicate readings for each standard and samples. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample is under the lowest limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

Typical data

As the OD values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should generate a standard curve for each experiment. Typical standard curve and data is provided below (for reference only).

Concentration(ng/mL)	O.D	Average
10	0.321 0.375	0.348
5	0.455 0.465	0.46
2.5	0.67 0.644	0.657
1.25	0.957 0.963	0.96
0.63	1.343 1.333	1.338
0.31	1.733 1.723	1.728
0.16	2.011 2.015	2.013
0	2.428 2.442	2.435



Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, mid-range and high level 5-Hydroxyeicosatetraenoic Acid were tested 20 times on one plate.
Inter-assay Precision (Precision between assays): 3 samples with low, mid-range and high level 5-Hydroxyeicosatetraenoic Acid were tested on 3 different plates, 20 replicates in each plate.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	0.54	0.92	4.64	0.53	0.84	4.76
Standard deviation	0.04	0.05	0.16	0.03	0.04	0.18
C V (%)	7.41	5.43	3.45	5.66	4.76	3.87

Recovery

The recovery of 5-Hydroxyeicosatetraenoic Acid spiked at three different levels in samples throughout the range of the assay was evaluated in various matrices.

Sample Type	Range (%)	Average Recovery (%)
Serum (n=5)	95-107	101
EDTA plasma (n=5)	92-105	99
Cell culture media (n=5)	88-100	94

Linearity

Samples were spiked with high concentrations of 5-Hydroxyeicosatetraenoic Acid and diluted with Reference Standard & Sample Diluent to produce samples with values within the range of the assay.

		Serum (n=5)	EDTA plasma (n=5)	Cell culture media (n=5)
1:2	Range (%)	83-98	94-108	86-98
	Average (%)	90	99	91
1:4	Range (%)	85-99	91-106	82-92
	Average (%)	90	98	87
1:8	Range (%)	82-93	91-106	86-98
	Average (%)	88	99	91
1:16	Range (%)	81-94	96-110	87-100
	Average (%)	86	101	93

9. Important General Notes:

Problem	Causes	Solutions
Poor standard curve	Inaccurate pipetting	Check pipettes.
	Improper standard dilution	Centrifuge the standard vial and ensure contents are dissolved thoroughly.
	Wells are not completely aspirated	Completely aspirate wells in between steps.
Low signal	Insufficient incubation time	Ensure sufficient incubation time.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrate to room temperature before use.
	Inadequate reagent volumes	Check pipettes and ensure correct preparation.
	Improper dilution	
	HRP conjugate inactive or TMB failure	Mix HRP conjugate and TMB = rapid color change.
Deep color but low value	Plate reader setting is not optimal	Verify the wavelength and filter setting on the Microplate reader.
		Preheat Microplate Reader
Large CV	Inaccurate pipetting	Check pipettes.
High background	Concentration of target protein is too high	Use recommended dilution factor.
	Plate is insufficiently washed	Review the manual for proper washing procedure. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Prepare fresh wash buffer.
Low sensitivity	Improper storage of the ELISA kit	All the reagents should be stored according to the instructions.
	Stop solution is not added	Stop solution should be added to each well before measurement.

Additional Notes:

1. Please wear lab coats, eye goggles and latex gloves for protection. Perform the experiment following the national security guidelines for biological laboratories, especially when using blood samples or other bodily fluids.

2. A freshly opened ELISA Plate may appear to have a water-like substance. This is normal and will not have any impact on the experimental results.

3. Do not reuse the reconstituted standard, biotinylated detection Ab working solution, concentrated HRP conjugate working solution. The unspent undiluted concentrated biotinylated detection Ab (100×) and other stock solutions should be stored according to the storage conditions in the above table.

4. The microplate reader should have a 450(±10 nm) filter installed and a detector that can detect this wavelength. The optical density should be within 0~3.5.

5. Do not mix or use components from other lots.

6. Change pipette tips in between adding standards, sample additions, and reagent additions. Also, use separate reservoirs for each reagent.

Declaration

1. Limited by current scientific technology, we can't conduct comprehensive identification and analysis on all the raw materials provided. (So, there might be some qualitative and technical risks for users using the kit.)

2. The final experimental results will be closely related to the validity of products, operational skills and the experimental environment. Please make sure that sufficient samples are available.

3. To get the best results, please only use the reagents supplied with this kit and strictly comply with the instructions.

4. Incorrect results may occur from incorrect reagent preparation and loading, as well as incorrect parameter settings of the Micro-plate reader. Please read the instructions carefully and adjust the instrument prior to the experiment.

5. Each kit passes a strict QC procedure. However, results from end users might be inconsistent with our data due to some variables such as transportation conditions, different lab equipment’s, and so on. Intra-assay variance among kits from different batches might also arise from the above reasons, too.

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