Technical Manual

VC (Vitamin C) ELISA Kit

- Catalogue Code: UNLE00001
- Competitive ELISA Kit
- Research Use Only

Contents

1. Key features and Sample Types

Aliases:

Vitamin C/Ascorbic Acid

Detection method:

Competitive

Sample Type:

Serum, Plasma, cell lysates. tissue homogenates, and other biological fluids

Reactivity:

General

Range:

625-40000 ng/ml

Sensitivity:

192.1 ng/ml

Storage:

2-8°C for 6 months. -20°C for 1 year. (For details see 2. Storage & Expiry).

Expiry:

See Kit Label

2. Storage & Expiry

Assay Genie ELISA Kits are shipped on ice packs. Date of expiration will be on the ELISA Box label.

1. *If the kit is opened, store the whole kit at 4°C. If the kit is not used up in 1 week. Store the Pre-Coated Microplate, Standard, Biotinylated-Ab and Streptavidin-HRP at -20°C, the rest of the reagents at 4°C, please use within 6 months.

*If the kit is not opened, store the whole kit: 4°C (short time storage, valid for 6 months); -20°C (long-term storage, valid for 1 year). Avoid repeated freeze-thaw cycles

2. Do not use the kit beyond the expiration date.

3. If the whole kit is stored at -20°C, place the kit at 4°C the day before the experiment.

4. After opening the package, please check that all components are complete.

5. The cap must be tightened to prevent evaporation and microbial contamination. The reagents volume is slightly more than the volume marked on labels, please use accurate measuring equipment and do not pour directly into the vial.

All kit components have been formulated and quality control tested to function successfully. Do not mix or substitute reagents or materials from other kits, detection effect of the kit will not be guaranteed if utilized separately or substituted

3. Description and Principle

This kit is based on Competitive-ELISA detection method. The microtiter plate provided in this kit has been pre-coated with analyte of interest (Vitamin C protein). During the reaction, the analyte in the sample or standard competes with a fixed amount of target on the solid phase supporter for sites on the biotinylated detection antibody (Biotinylated-Conjugate) specific to Vitamin C. Excess conjugate and unbound sample or standard are washed from the plate, and HRP-Streptavidin (SABC) is added to each microplate well and incubated. TMB substrate solution is then 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 450nm. There is an inverse correlation between the OD value of the sample and the concentration of Vitamin C. The concentration of target in the samples is then determined by comparing the OD of the samples to the standard curve.

4. Kit Contents

Each kit contains reagents for either 48 or 96 assays including:

No.	Component	48-Well Kit	96-Well Kit	Storage
1	ELISA Microplate (dismountable)	8 x 6	8 x 12	2-8°C/-20°C
2	Lyophilized Standard	1 vial	2 vials	2-8°C/-20°C
3	Sample/Standard Dilution Buffer	10 mL	20 mL	2-8°C/-20°C
4	Biotinylated-Conjugate (100X)	30 uL	60 uL	2-8°C/-20°C
5	Biotinylated-Conjugate Diluent	5 mL	10 mL	2-8°C/-20°C
6	Streptavidin-HRP (100X)	60 uL	120 uL	2-8°C/-20°C (Avoid Direct Light)
7	HRP Diluent	6 mL	12 mL	2-8°C/-20°C
8	TMB Substrate Solutions	6 mL	10 mL	2-8°C/-20°C (Avoid direct light)
9	Stop Regeant	3ml	6ml	2-8°C/-20°C
10	Wash Buffer (25x)	10 mL	20 mL	2-8°C/-20°C
11	Plate Covers	1 piece	2 pieces	
12	Manual	1	1	

Additional materials required:

1. 37°C incubator
2. Plate Reader with 450nm filter
3. Precision pipettes and disposable pipette tips
4. Distilled water
5. Disposable tubes for sample dilution
6. Absorbent paper
7. High-speed centrifuge

Precautions:

1. To identify the concentration of your target, a pilot experiment using standards and a small number of samples is recommended.

2. Ensure unopened and unused plate is kept dry to avoid contamination.

3. Before using the kit, centrifuge tubes to spin down standard & antibodies.

4. Avoid light for storage of TMB reagents.

5. Wash steps are critical for the success of the assay, deviations from wash steps may cause false positives and result in a high background.

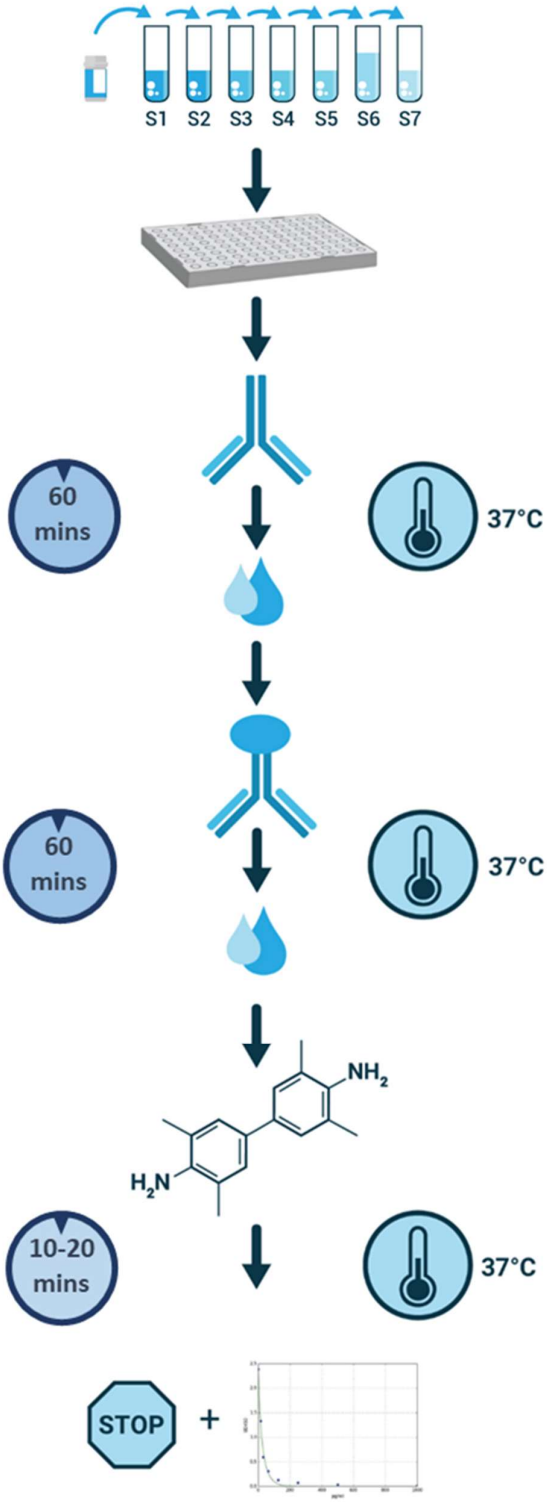
6. Duplicate wells are recommended for both standard and sample testing.

7. Do not let the microplate wells dry during assay. Dry plates will inactivate active components.

8. Do not reuse tips and tubes to avoid cross contamination.

9. Avoid using the reagents from different batches together.

5. Workflow Overview



Prepare plate standards and reagents

Add 50µl of samples, standards and controls. Immediately add 50µl Biotinylated-Conjugate working solution.

Wash plate X3

Add 100µl Streptavidin-HRP working solution.

Wash plate X5

Add 90µl TMB Substrate.

Add 50µl STOP solution and read absorbance @450nm

6. Sample Preparation

General considerations: According to best practices, extract protein & perform the experiment as soon as possible after sample collection. Alternatively, store the extracts at the designated temperature (-20°C/-80°C). For optimal results, avoid repeated freeze-thaw cycles.

Serum: Samples should be collected into a serum separator tube. After clotting for 2 hours at room temperature or overnight at 4°C, and then centrifuging at 1000 × g for 20 minutes. Assay freshly prepared serum immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples at 1000 × g and 2-8°C for 15 minutes within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze-thaw cycles.

Note: Over haemolyzed samples are not suitable for use with this kit.

Adherent and Suspension Cell Culture: Use three T25 flasks or one T27 flask for cell culture. Cell number is ($1x10^7$).

Cell Lysate Preparation: Cells need to be lysed before assaying according to the following directions.

1. Adherent cells should be washed by pre-cooled PBS gently, and then be detached with trypsin, and collect them by centrifugation at 1000 × g for 5 minutes (suspension cells can be collected by centrifugation directly).

2. Wash cells 3 times in pre-cooled PBS.

3. Then, resuspend the cells in fresh lysis buffer with concentration of 107 cells/mL. If it is necessary, the cells could be subjected to ultrasonication until the solution is clear.

4. Centrifuge at 1500 × g for 10 minutes at 2-8°C to remove cellular debris. Assay immediately or store in aliquots at ≤ -20°C.

Urine: Collect the first urine of the day (mid-stream) and discharge it directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Saliva: Collect saliva using a collection device or equivalent. Centrifuge samples at 1000 × g at 2-8°C for 15 minutes. Remove particulates and assay immediately or store samples in aliquot at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Cell culture supernatants and other Biological Fluids: Centrifuge samples at 1000 × g for 20 minutes. Collect the supernatant and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze-thaw cycles.

Tissue Homogenates: The preparation of tissue homogenates will vary depending upon tissue type.

1. Rinse the tissues in pre-cooled PBS to completely remove excess blood, and weigh them before homogenization.

2. Mince the tissues to small pieces and homogenize them in fresh lysis buffer (different lysis buffer needs to be chosen based on subcellular location of the target protein) (PBS can be used as the lysis buffer of most tissues) (w:v = 1:9, e.g. 900 µL lysis buffer is added in 100 mg tissue sample) with a glass homogenizer on ice (micro tissue grinders, too).

3. Ultrasound the obtained suspension with an ultrasonic cell disrupter until the solution is clear.

4. Then, centrifuge the homogenates for 5 minutes at 10000 × g and collect the supernatant and assay immediately or store in aliquots at ≤ -20°C.

*Note: Tissue homogenates are recommended to be tested for protein concentration at the same time to obtain a more accurate concentration of the test substance per mg of protein.

Notes

1. 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 bio-activity and contamination. Avoid repeated freeze-thaw cycles.

2. Sample hemolysis will influence the result, so it should not be used.

3. When performing the assay, bring samples to room temperature.

4. If the concentration of the test material in your sample is higher than that of the Standard product, please make the appropriate multiple dilutions according to the actual situation (it is recommended to do preliminary experiment to determine the dilution ratio.

7. Standard and Reagent Preparation

Manual Washing

Discard the solution in the plate without touching the side of the wells. Clap the plate on absorbent filter paper or other absorbent material. Fill each well completely with 200 µl wash buffer and soak for 1 to 2 mins, then aspirate contents from the plate, and clap the plate on absorbent filter paper or other absorbent material. Repeat this procedure for the designated number of washes.

Automated Washing

Aspirate all wells, then wash plate with 200 µl wash buffer. After the final wash, invert plate, and clap the plate on absorbent filter paper or other absorbent material. It is recommended that the washer is set for a soaking time of 1 minute (Note: set the height of the needles; be sure the fluid can be taken up completely).

Sample Dilution Guidelines

Determine the concentration of the target protein in the test sample and then select the optimal dilution factor to ensure the target protein concentration falls within the optimal detection range of the kit. Dilute the samples with the dilution buffer provided with the kit. Several dilution tests may be required to achieve the optimal results. The test samples must be well mixed with the dilution buffer. Standard and sample dilution should be performed before starting the experiment.

Note: Matrix components in the sample will affect test results, samples need to be diluted at least ½ with Sample Dilution Buffer before testing.

Reagent Preparation

Bring all reagents and samples to room temperature 20 minutes before use. Make sure all components are dissolved and mixed well before using the kit.

1. Wash Buffer:

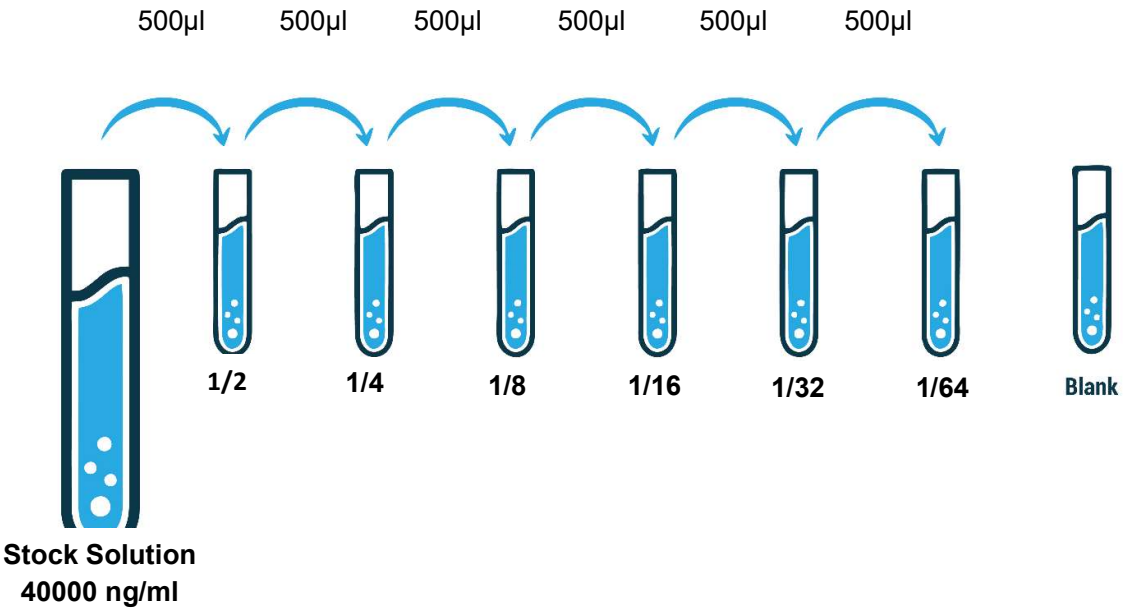
Dilute the 25 × Wash Buffer into 1× Wash Buffer with double distilled water. If crystals form in the concentrate (25×), heat it to room temperature (the heating temperature should not exceed 40°C), gently mix until crystals are completely dissolved.

2. Standard Working Solution:

1. Centrifuge the Standard at 1000 × g for 1 minute. Reconstitute the Standard with 1.0 mL of Sample/Standard Dilution Buffer, kept for about 10 minutes at room temperature, shake gently (not to foam). The concentration of the standard in the stock solution is 40000 ng/mL.

2. Label 7 Eppendorf tubes with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank respectively. Aliquot 500μl of the Sample dilution buffer into each tube. Add 500μl of the above (Stock Solution) standard solution into 1st tube and mix thoroughly. Transfer 500μl from 1st tube to 2nd tube and mix thoroughly. Transfer 500μl from 2nd tube to 3rd tube and mix thoroughly, and so on. Sample dilution buffer is used as blank control.

DILUTION SERIES



Note: Prepare the standard solution 15 minutes before the test. In order to guarantee the experimental results validity, please use a new standard solution for each experiment.

3. Preparation of Biotinylated-Conjugate working solution:

1. Calculate the total volume of the working solution: 50µl / well × quantity of wells (Allow 100-200µl more than the total volume).
2. Briefly spin or centrifuge the stock Biotinylated-Conjugate before use. Dilute it to the working concentration 100-fold with Biotinylated-Conjugate Diluent. For example, 10 µL of Biotinylated-Conjugate with 990 µL of Biotinylated-Conjugate Diluent.

4. Preparation of HRP-Streptavidin working solution:

1. Calculate the total volume of the working solution: 100µl / well × quantity of wells. (Allow 100-200µl more than the total volume)
2. Briefly spin or centrifuge the stock HRP-Streptavidin before use. Dilute it to the working concentration 100-fold with HRP dilution buffer. For example, 10 µL of HRP with 990 µL of HRP diluent.

5. TMB Substrate Solution:

Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again. TMB Substrate Solution is light-sensitive, avoid prolonged exposure to light. Dispense the TMB Substrate Solution within 15 minutes following the washing of the microtiter plate. In addition, avoid contact between TMB Substrate Solution and metal to prevent color development. TMB is contaminated if it turns blue color before use and should be discarded. TMB is toxic, avoid direct contact with hands.

8. Assay Procedure

1. **Set standard, test sample and control (blank) wells** on the pre-coated plate respectively, and then, record their positions. It is recommended to measure each standard and sample in duplicate**.**
2. **Add Sample and Biotinylated-Conjugate:** Add 50μL of Standard, Blank or Sample per well. The blank well is added with Sample Dilution Buffer. Immediately add 50 μL of Biotinylated-Conjugate working solution to each well. Cover with the plate sealer provided. Gently tap the plate to ensure thorough mixing. Incubate for 60 minutes at 37°C. (Solutions are added to the bottom of micro-ELISA plate well, avoid touching plate walls and foaming).
3. **Wash:** Aspirate each well and wash, repeating the process X3 according to instructions. **Let the wash buffer stay in the wells for 1-2 minutes each time.** After final wash, remove any remaining wash buffer by aspirating or decanting
4. **Streptavidin-HRP:** Add 100μL of Streptavidin-HRP working solution to each well.
5. **Incubate:** Cover with a Plate sealer. Incubate for 60 minutes at 37°C.
6. **Wash:** Repeat the aspiration/wash process X5 according to instructions. **Let the wash buffer stay in the wells for 1-2 minutes each time.**
7. **TMB Substrate:** Add 90μL of TMB Substrate to each well. Cover with a plate sealer. Incubate for about 10-20 minutes in the dark at 37°C. Protect from light.

   Note: The reaction time can be shortened or extended according to the actual color change, but not more than 30 minutes. When apparent gradient appears in standard wells, you can terminate the reaction. The liquid will turn blue by the addition of TMB Substrate Solution. Preheat the Microplate Reader for about 15 minutes before OD measurement.
8. **Stop:** Add 50μL of Stop Solution to each well. Wells will turn to yellow immediately. The adding order of stop solution should be as the same as the substrate solution.
9. **OD Measurement:** Determine the optical density (OD Value) of each well at once, using a microplate reader set to 450 nm. You should open the microplate reader ahead, preheat the instrument, and set the testing parameters. Wipe off any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid.

9. Data Analysis

This assay employs the competitive inhibition enzyme immunoassay technique, so there is an inverse correlation between VC concentration in the sample and the assay signal intensity. Regarding calculation, the standard curve can be plotted as the O.D.450 of each standard solution (Y) vs, the respective concentration of the standard solution (X). The target concentration of the samples can be interpolated from the standard curve. Curve Expert 1.3 or 1.4 is recommended for data analysis.

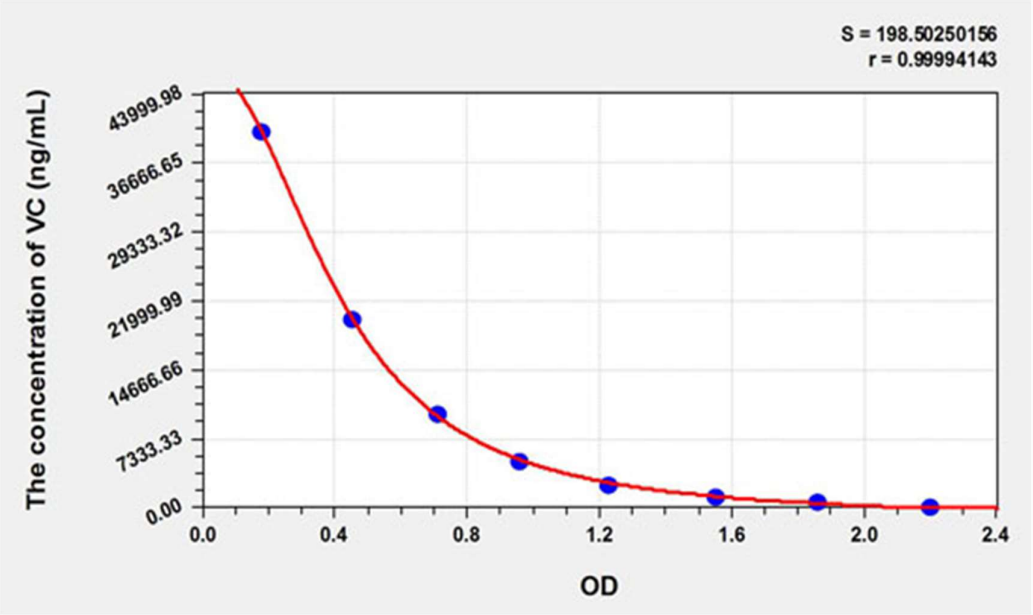
Note: If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

10. Typical Data & Standard Curve

Standard Curve

Results of a typical standard run of an ELISA kit are shown below. This standard curve was generated at our lab for demonstration purpose only. Each user should obtain their own standard curve as per experiment.

Concentration (ng/mL)	OD
40000	0.195
20000	0.467
10000	0.722
5000	0.967
2500	1.236
1250	1.557
625	1.861
0	2.197



Note: this graph is for reference only

Specificity

This assay has high sensitivity and excellent specificity for detection of Vitamin C. No significant cross-reactivity or interference between Vitamin C and analogues was observed.

Note: Limited by current skills and knowledge, it is difficult for us to complete the cross-reactivity detection between Vitamin C and all the analogues, therefore, cross reaction may still exist.

Recovery

Matrices listed below were spiked with certain level of recombinant VC and the recovery rates were calculated by comparing the measured value to the expected amount of VC in samples.

Matrix	Recovery range	Average
Serum (*n* = 5)	85-97%	91%
EDTA plasma (*n* = 5)	91-105%	98%
Heparin plasma (*n* = 5)	81-95%	101%

Linearity

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of VC and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1:2	1:4	1:8	1:16
Serum (*n* = 5)	93-101%	93-102%	79-94%	87-102%
EDTA plasma (*n* = 5)	95-104%	93-102%	79-94%	87-98%
Heparin plasma (*n* = 5)	86-93%	86-97%	85-103%	89-97%

Precision

- **Intra-Assay:** CV<8%

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

- **Inter-Assay:** CV<10%

Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

11. Troubleshooting

High background/Non-specific staining

Description of results	Possible reason	Recommendations and precautions
After termination, the whole plate results show a uniform yellow or light color; or the Standard curve is linear but the background is too high	The yellowing of the whole plate may be caused by wrong addition of other reagents	Check the components and lot numbers of the reagents before the experiment, and confirm that all components belong to the corresponding kit. Reagents from different kits or different lot numbers cannot be mixed.
	ELISA plate was not washed sufficiently	Make sure that the same amount of Washing Solution is added to each microwell during the washing process. After washing, press the ELISA plate firmly on the absorbent paper to remove the residual buffer.
	Incubation time too long	Please strictly follow the steps of the manual
	Streptavidin-HRP contaminates the tip and TMB container or positive control contaminates the Pre-coated Microplate	When absorbing different reagents, the tips should be replaced. When configuring different reagent components, different storage vessels should be used. Please use a pipette during operation.
	Biotinylated-Conjugate or Streptavidin-HRP concentration too high	Check whether the concentration calculation is correct or use after further dilution.
	Substrate exposure or contamination prior to use	Store in the dark at all times before adding substrate.
	Color development time is too long	Please strictly follow the steps of the manual.
	The wrong filter was used when the absorbance value was read	When TMB is used as the substrate, the absorbance should be read at 450 nm.

NO color plate

Description of results	Possible reason	Recommendations and precautions
After the color development step, all wells of the ELISA plate are colorless; the positive control is not obvious	Mixed use of component reagents	Please read labels clearly when preparing or using
	In the process of plate washing and sample addition, the enzyme marker is contaminated and inactivated, and loses its ability to catalyze the color developing agent	Confirm that the container holding the ELISA plate does not contain enzyme inhibitors (such as NaN_3, etc.), and confirm that the container for preparing the Wash Solution has been washed.
	Missing a reagent or a step	Review the manual in detail and strictly follow the operating steps

Light color

Description of results	Possible reason	Recommendations and precautions
The Standard is normal, the color of the sample is light	The sample uses NaN_3 preservative, which inhibits the reaction of the enzyme	Samples cannot use NaN_3
	The sample to be tested may The standard is normal, the color of the sample is lightnot contain strong positive samples, so the result may be normal	In case of doubt, please test again.
The visual result is normal, but the reading value of the microplate reader is low	Wrong filter used for absorbance reading	When TMB is used as the substrate, the absorbance should be read at 450 nm.

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