ELK Biotechnology

EasyStep Rat 25-OH-D(25 Hydroxy Vitamin D) ELISA Kit

Cat: ELK053ES

For research use only. Not intended for diagnostic use.

Assay Type: Competitive Inhibition

Sensitivity: 7.7 ng/mL

Detection Range: 78.13-5000 ng/mL

Specificity: This assay has high sensitivity and excellent specificity for detection of Rat 25-OH-D. No significant cross-reactivity or interference between Rat 25-OH-D and analogues was observed.

Please refer to the outer packaging label of the kit for the specific shelf life.

KIT Components

Reagents	Quantity		Storage Condition
	64T	112T	
Pre-Coated Microplate	6 strips x 8 wells	12 strips x 8 wells	4°C/-20°C
Standard Microplate	2 strips x 8 wells	2 strips x 8 wells	4°C/-20°C
HRP Conjugate Antibody (100×)	35 µL	70 µL	4°C/-20°C(store in dark)
Standard/Sample Diluent Buffer	12 mL	24 mL	4°C/-20°C
HRP Conjugate Diluent	5 mL	10 mL	4°C/-20°C
Wash Buffer (25×)	12 mL	24 mL	4°C/-20°C
TMB Substrate Solution	7 mL	12 mL	4°C/-20°C (store in dark)
Stop Reagent	4 mL	7 mL	4°C/-20°C
Plate Covers	1 piece	2 pieces	4°C/-20°C
Instruction Manual	1	1	RT

Special Explanation

1. *If the kit is opened, store Standard Microplate at -20°C, the rest reagents at 4°C. If the kit is not used up in 1 week, please store Standard Microplate,Pre-Coated Microplate and HRP Conjugate Antibody at -20°C, the rest reagents at 4°C, please used up 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.

Materials Required, Not Supplied

1. Microplate reader capable of measuring absorbance at 450 ± 10 nm.
2. High-speed centrifuge.
3. Electro-heating standing-temperature cultivator and Microplate oscillator.
4. Absorbent paper.
5. Double distilled water or deionized water.
6. Single or multi-channel pipettes with high precision and disposable tips.
7. Precision pipettes to deliver 2 µL to 1 mL volumes.

Safety Notes

1. This kit is only used for lab research and development and should not be used for human or animals.
2. Reagents should be regarded as hazardous substances and should be handled carefully and correctly.
3. Gloves, lab coats, and goggles should always be worn to avoid skin and eyes coming into contact

with Stop Solution and TMB. In case of contact, wash thoroughly with water.

Test Principle

This assay employs the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with Rat 25-OH-D antigen, and the Rat 25-OH-D standard plate wells that pre-coated using protein-related techniques are provided separately. Standard/Sample Diluent Buffer or samples are added to the appropriate microtiter plate wells ,then added a HRP-conjugated antibody specific to Rat 25-OH-D to each microplate well and incubated . After TMB substrate solution is added, the enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ±10nm. The concentration of Rat 25-OH-D in the samples is then determined by comparing the OD of the samples to the standard curve.

Sample Collection and Storage

Serum - Samples should be collected into a serum separator tube. After clotting for 2 hours at room temperature or overnight at 4℃, and then centrifuging at 1000 × g for 20 minutes. Assay freshly prepared serum immediately or store samples in aliquot at -20℃ or -80℃ 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℃ for 15 minutes within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquot at -20℃ or -80℃ 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 homogenized 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℃.

*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. For protein detection, you can purchase our product: **BC016, BCA Protein concentration determination kit**.

Cell lysates - 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 (PBS) with concentration of 10^7 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℃ to remove cellular debris. Assay immediately or store in aliquots at ≤ -20 ℃.

Urine - Collect the first urine of the day (mid-stream) and discharge it directly into a sterile container. Centrifuge at 2000 × g for 15 minutes at 2-8°C 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.

Feces - Dry feces were collected as much as possible, weighing more than 50 mg. The feces were washed three times with PBS (w:v = 1:9, e.g. 900 μL lysis buffer is added in 100 mg feces), sonicated (or mashed) and centrifuged at 5000×g for 10 minutes, where the supernatant was collected for testing.

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.

Cerebrospinal fluid (CSF) - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤-20°C. Avoid repeated freeze-thaw cycles.

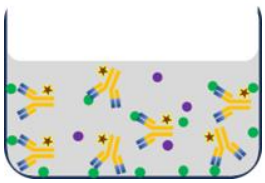
Sample Dilution Proposal

Normal Rat serum and plasma samples are recommended for Original solution testing.

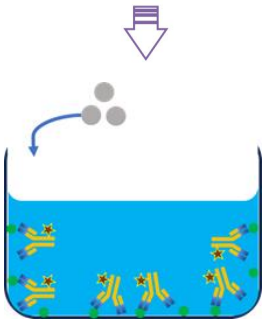
Notes

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 (≤ 6 months) to avoid loss of bioactivity and contamination. Avoid repeated freeze-thaw cycles.
2. The sample should be clear and transparent, and the suspended matter should be removed by centrifugation.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.

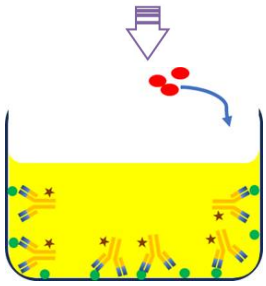
Summary



1.After the kit is equilibrated at room temperature, add 50µL Standard/Sample Diluent Buffer to each Standard well， and add 50 µL sample to the sample well, Immediately add 50 µL 1× HRP Conjugate Antibody Working Solution to each well, Incubate at 37°C on Microplate oscillator for 60 min .



2.Discard the liquid in the plate, add 200 µL 1× Wash Buffer to each well, and wash the plate 5 times. After pat it dry against clean absorbent paper, add 90 µL TMB Substrate Solution to each well, incubate at 37°C for 20 minutes in the dark.



3.Add 50 µL Stop Solution to each well, shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm immediately, calculation of the results.

Reagent Preparation

1. Bring all kit components and samples to room temperature (18-25℃) before use.Make sure all components are dissolved and mixed well before using the kit.
1. If the kit will not be used up in 1 time, please only take out strips and reagents for present experiment, and save the remaining strips and reagents as specified.
2. Dilute the 25× Wash Buffer into 1× Wash Buffer with double-distilled Water.
3. Samples are diluted with Standard & Sample Diluent according to pre-test or sample dilution Proposal.
4. **1× HRP Conjugate Antibody -** Briefly spin or centrifuge the stock HRP Conjugate Antibody before use. Before the experiment, the dosage required for the experiment (50 µL/ well, the actual configured total amount must be 50-100µL greater than the calculated value) was calculated, Dilute HRP Conjugate Antibody to the working concentration 100-fold with HRP Conjugate Diluent. The dilution principle is to take 1 µL concentrated HRP conjugated antibody and add it to 99 µL HRP Conjugate Diluent and mix well.
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.

Notes

1. After receive the kit, please store the reagents according to the instructions. The plates can be disassembled to single strips. Please use it in batches on demands.
2. The test tubes, pipette tips and reagents used in the experiment are all disposable and are strictly prohibited from being reused; otherwise the experiment results will be affected. Kit reagents of different batches cannot be mixed (except TMB, Washing Buffer and Stop Reagent).
3. The standard strips are provided separately that contains white solid standard.Re-seal the unused standard slats and place them at -20℃ for use as soon as possible.Place the standard slats upwards as far as possible. When opening the cover, check whether there is a solid standard on the plug. If so, the solid standard needs to be moved into the corresponding well with a pipette.
4. If the Standard/Sample Diluent Buffer is added to the standard well ,but the white solid standard on the wall is not dissolved , it is necessary to flush the diluent in the appropriate well with a pipette to completely dissolve the standard product.

5. **HRP Conjugate Antibody** is small in volume and may be scattered in various parts of the tube during transportation. Please centrifuge at 1000 × g for 1 minute before use. Then, carefully pipette 4-5 times to mix the Solution. Please configure the **HRP Conjugate Antibody** Working Solution according to the required amount, and use the corresponding Dilution Solution, cannot be mixed used.
6. When incubating antigen and HRP conjugate antibody, it is necessary to use a micro-plate oscillator to oscillate the Microplate. If the plate is not oscillated, the reaction will be inadequate, and the OD value will decrease overall. The amplitude should not exceed half the height of the well, Too much oscillation would causes the background to rise.
7. Bring all reagents to room temperature (18-25℃) before use. If crystals form in the concentrate (25×), it is a normal phenomenon. Heat it to room temperature (the heating temperature should not exceed 40℃), gently Mix until crystals are completely dissolved.
8. Firstly, add the Standard/Sample Diluent Buffer to the required standard wells to dissolve the standard , and then add sample to the sample wells . The sample addition needs to be rapid. Each sample addition should preferably be controlled within 10 minutes. To ensure experimental accuracy, it is recommended to test duplicate wells, and when pipetting reagents, keep a consistent order of additions from 1 well to another, this will ensure the same incubation time for all wells.
9. During the washing process, the residual washing liquid in the reaction well should be patted dry on absorbent paper. Do not put the paper directly into the reaction well to absorb water. Before reading, pay attention to remove the residual liquid and fingerprints at the bottom, so as not to affect the microplate reader reading.
10. 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.
11. Bacterial or fungal contamination of either samples or reagents or cross-contamination, between reagents may cause erroneous results.

Samples Preparation

1. Equilibrate all materials and prepared reagents to room temperature prior to use. Prior to use, mix all reagents thoroughly taking care not to create any foam within the vials.

2. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
3. Please predict the concentration before assaying. If values for these are not within the range of the Standard curve, users must determine the optimal sample dilutions for their particular experiments.

Assay Procedure

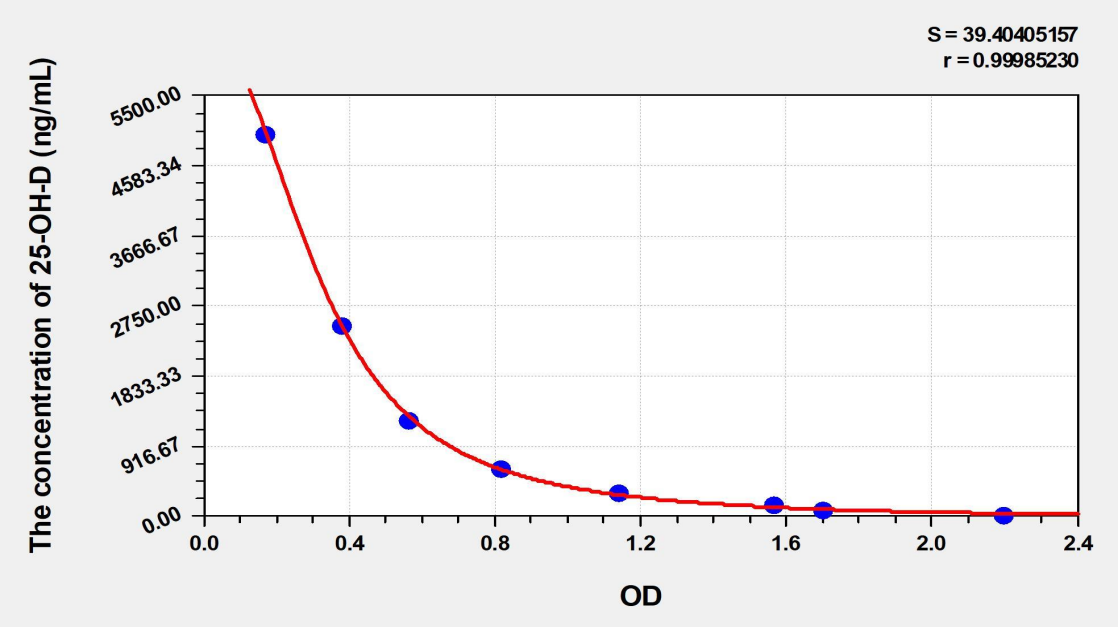
1. Before the experiment starts, all reagents should be balanced to room temperature, and all reagents should be prepared in advance. When diluting the reagent or sample, it is necessary to mix, and try to avoid foaming when mixing. If the sample concentration is too high, dilute it with a sample diluent to make the sample conform to the detection range of the kit.
2. Place the labeled standard strip in the frame of the Microplate, add 50µL Standard/Sample Diluent Buffer to each standard well, add 50µL sample to the sample wells (if the sample needs to be diluted, please refer to the sample dilution suggestion), pay attention to no bubbles, add the sample to the bottom of the Microplate well when adding the sample, do not touch the wall of the well. Then, each well was immediately added with 50µL HRP conjugate antibody working solution(Note: The tips don't touch the liquid in the wells when adding HRP conjugate antibody working solution), Cover the microplate with Plate Cover and oscillate the Microplate with the oscillator at 500 RPM (other horizontal oscillators should adjust their own speed to ensure that the solution per well did not exceed half height of the wells and could be mixed), and incubated at 37°C for 60 minutes
3. Discard the liquid in the wells and wash the plate 5 times. Wash each well with 200µL of washing solution, soak for 1-2 minutes each time, and shake off the liquid in the plate (or wash the plate with a plate washer). After the last wash, pat the plate dry on absorbent paper.
4. Add 90 µL of TMB Substrate Solution to each well. Cover with a new Plate Cover. Incubate for 20 minutes at 37℃ (Don't exceed 30 minutes) in the dark. The liquid will turn blue by the addition of TMB Substrate Solution. Preheat the Microplate Reader for about 15 minutes before OD measurement.
5. Add 50 µL of Stop Reagent to each well. The liquid will turn yellow by the addition of Stop Reagent. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The insertion order of the Stop Reagent should be the same as that of the TMB Substrate Solution.
6. 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. Then, run the microplate reader and conduct measurement at 450 nm immediately.

Calculation of Results

This assay employs the competitive inhibition enzyme immunoassay technique, so there is an inverse correlation between Rat 25-OH-D concentration in the sample and the assay signal intensity. Average the duplicate readings for each standard, control, and samples. Create a standard curve with the Rat 25-OH-D concentration on the y-axis and absorbance on the x-axis. Draw the best fit straight line through the standard points and it can be determined by regression analysis. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor. Using some plot software, for instance, curve expert.

Concentration (ng/mL)	OD
5000	0.189
2500	0.397
1250	0.583
625	0.833
312.5	1.157
156.25	1.583
78.13	1.715
0	2.211



Note: this graph is for reference only

Precision

Intra-assay Precision (Precision within an assay)： **CV% < 8%**

Three samples of known concentration were tested twenty times on 1 plate to assess intra-assay precision.

Inter-assay Precision (precision between assays)： **CV% < 10%**

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

Recovery

Matrices listed below were spiked with certain level of recombinant Rat 25-OH-D and the recovery rates were calculated by comparing the measured value to the expected amount of Rat 25-OH-D in samples.

Matrix	Recovery range	Average
Serum (*n* = 5)	85-97%	91%
EDTA plasma (*n* = 5)	91-105%	98%
Heparin plasma (*n* = 5)	95-107%	101%

Linearity

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of Rat 25-OH-D 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)	85-107%	84-98%	95-103%	86-98%
EDTA plasma (*n* = 5)	86-93%	91-105%	87-98%	99-102%
Heparin plasma (*n* = 5)	85-97%	87-96%	86-98%	91-103%

Sample Value

This kit is applied to detect 8 of Serum samples of healthy Rat.

Sample Type	Sample Number	Range	Mean	Positive Rate
Serum	8	73.28-86.99 ng/mL	78.75 ng/mL	100%

Note: This sample range is not physiological. The concentration range of samples varies depending on species, sample preparation, and testing personnel and equipment. The above data is for reference only.

Declaration

1. The kit may not be suitable for special experimental samples where the validity of the experiment itself is uncertain, such as gene knockout experiments.
2. Certain natural or recombinant proteins, including prokaryotic and eukaryotic recombinant proteins, may not be detected because they do not match the detection antibody and capture antibody used in this product.
3. This kit is not compared with similar kits from other manufacturers or products with different methods to detect the same object, so inconsistent test results cannot be ruled out.

Analysis of Common Problems and Causes of ELISA Experiment

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.
	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 not 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.
The color of all the plate is light	No microplate oscillator is used	A microplate oscillator is required

Description of results	Possible reason	Recommendations and precautions
All wells, including Standard and Samples, are lighter in color	Insufficient incubation time	Timer accurate timing
	Insufficient color reaction	Usually 15 - 30 minutes
	The number of washings increases, and the dilution ratio of the concentrated lotion does not meet the requirements	Reduce the impact of washing, dilute the concentrated lotion and washing time according to the manual, and accurately record the washing times and dosage.
	Distilled water quality problem	The prepared lotion must be tested to see if the pH value is neutral.
	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.), confirm that the container for preparing the Washing Solution has been washed, and confirm that the purified water for preparing the Washing Solution meets the requirements and is not contaminated.
	The kit has expired or been improperly stored	Please use it within the expiration and store it in accordance with the storage conditions recommended in the manual to avoid contamination.
	Reagents and samples are not equilibrated before use	All reagents and samples should be equilibrated at room temperature for about 30 minutes.
	Insufficient suction volume of the pipette, too fast discharge of pipetting suction, too much liquid hanging on the inner wall of the tip or the inner wall is not clean.	To calibrate the pipette, the tips should be matched, each time the tips should fit tightly, the pipetting should not be too fast, and the discharge should be complete. The inner wall of the tips should be clean, and it is best to use it once.

Description of results	Possible reason	Recommendations and precautions
Poor repeatability	Incubation temperature constant temperature effect is not good	Keep the temperature constant to avoid the local temperature being too high or too low
	When adding liquid, too much remains on the medial wall of wells	When adding liquid, the tip should try to add liquid along the bottom of the medial wall of wells without touching the bottom of the hole.
	Reuse of consumables	The tips should be replaced when different reagents are drawn, and different storage vessels should be used when configuring different reagent components.
	The bottom of the microwell is scratched or there is dirt	Be careful when operating, be careful not to touch the bottom and wipe the bottom of the microplate to remove dirt or fingerprints.
		Technical repetition of the same sample for 3 times, including more than 2 approximate values.
	Cross-contamination during sample addition	Try to avoid cross-contamination when adding samples
The color of plate is chaotic and irregular	Cross-contamination from manual plate washing	When washing the plates by hand, the first 3 injections of the lotion should be discarded immediately, and the soaking time should be set for the next few times to reduce cross-contamination.
	Cross-contamination when clapping	Use a suitable absorbent paper towel when clapping the plate, do not pat irrelevant substances into the well of the plate, and try not to pat in the same position to avoid cross-contamination.

Description of results	Possible reason	Recommendations and precautions
The color of plate is chaotic and irregular	The liquid filling head of the plate washer is blocked, resulting in unsatisfactory liquid addition or large residual amount of liquid suction, resulting in the color of plate is chaotic and irregular	Unblock the liquid addition head, so that each well is filled with washing liquid when washing the plate and the residual amount should be small when aspirating liquid.
	Incomplete centrifugation of the sample, resulting in coagulation in the reaction well or interference of sediment or residual cellular components	Serum plasma should be fully centrifuged at 3000 rpm for more than 6 minutes
	The sample is stored for too long time, resulting in contamination.	Samples should be kept fresh or stored at low temperature to prevent contamination
	Incorrect preparation of Washing Solution or direct misuse of concentrated Washing Solution	Please configure according to the manual