9051 Mira Mesa Blvd, #910094
San Diego, CA 92191
Tel: 760-390-3989

Simian GJb1(Gap Junction Protein Beta 1) ELISA Kit

ADMEbio Catalog Number: EKEL15274MK

For Research Use Only. Not for use in diagnostic procedures.

Product Page: www.admebio.com/products/EKEL15274MK

Assay Type	Sandwich
Detectable Sample Type	Tissue homogenates and other biological fluids.
Sensitivity	0.113 ng/mL
Detection Range	0.32-20 ng/mL
Specificity	This assay has high sensitivity and excellent specificity for detection of Simian GJb1. No significant cross-reactivity or interference between Simian GJb1 and analogues was observed.

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

KIT Components

	Quantity		
Reagents	**48T**	**96T**	**Storage Condition**
Pre-Coated Microplate	6 strips x 8 wells	12 strips x 8 wells	4°C/-20°C
Standard (Lyophilized)	1 vial	2 vials	4°C/-20°C
Biotinylated Antibody (100×)	60 µL	120 µL	4°C/-20°C
Streptavidin-HRP (100×)	60 µL	120 µL	4°C/-20°C
Standard/Sample Diluent Buffer	10 mL	20 mL	4°C/-20°C
Biotinylated Antibody Diluent	6 mL	12 mL	4°C/-20°C
HRP Diluent	6 mL	12 mL	4°C/-20°C
Wash Buffer (25×)	10 mL	20 mL	4°C/-20°C
TMB Substrate Solution	6 mL	10 mL	4°C/-20°C (store in dark)
Stop Reagent	3 mL	6 mL	4°C/-20°C
Plate Covers	1 piece	2 pieces	RT

Special Explanation

1. *If the kit has been opened, store the entire kit at 4°C. If the kit is not used up within one week, store the Pre-Coated Microplate, Standard, Biotinylated Antibody, and Streptavidin-HRP at -20°C, and store the remaining reagents at 4°C. Use all reagents within six 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.

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

The test principle applied in this kit is Sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to Simian GJb1. Standards or samples are added to the appropriate microtiter plate wells then with a biotin-conjugated antibody specific to Simian GJb1. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain Simian GJb1, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. 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 Simian GJb1 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°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.

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

9051 Mira Mesa Blvd, #910094
San Diego, CA 92191
Tel: 760-390-3989

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°C.

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°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 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

1. Fresh, normal serum/plasma samples are recommended for Original solution testing.

2. Due to individual differences, the recommended dilution Ratio are for reference only, it is recommended to do preliminary experiment to determine the dilution ratio.

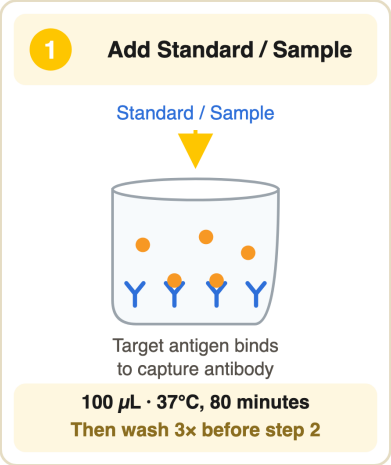
Notes

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.

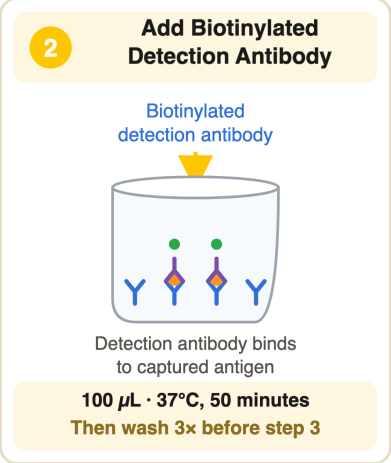
2. Sample hemolysis will influence the result, so it should not be used.

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

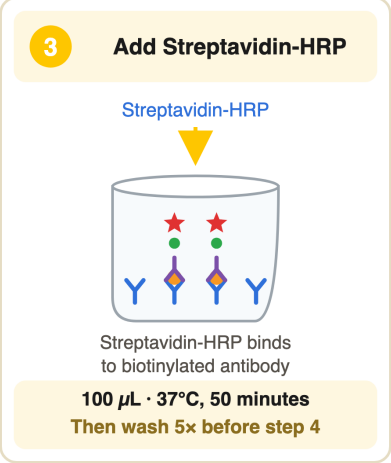
Summary



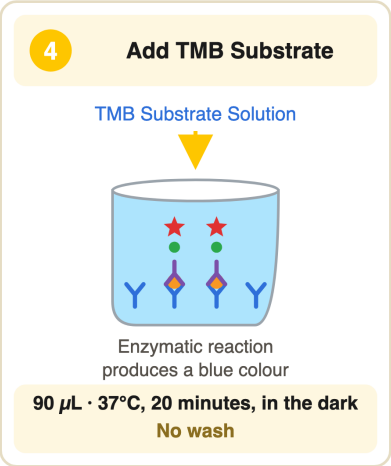
1. After the kit is equilibrated at room temperature, add 100 µL of Standard Working Buffer (gradually diluted according to the instructions) or 100 µL of sample to each well, and incubate at 37°C for 80 minutes.



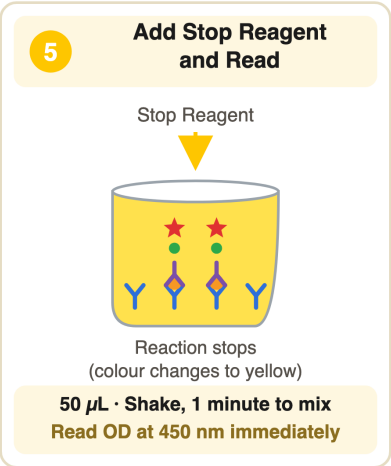
2. Discard the liquid in the plate, add 200 µL 1× Wash Buffer to each well, and wash the plate 3 times. After pat it dry against clean absorbent paper, add 100 µL Biotinylated Antibody Working Solution (1×) to each well, incubate at 37°C for 50 minutes.



3. Discard the liquid in the plate, add 200 µL 1× Wash Buffer to each well, and wash the plate 3 times. After pat it dry against clean absorbent paper, add 100 µL 1× Streptavidin-HRP Working Solution to each well, incubate at 37°C for 50 minutes.



4. 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.



5. 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°C) before use.Make sure all components are dissolved and mixed well before using the kit.

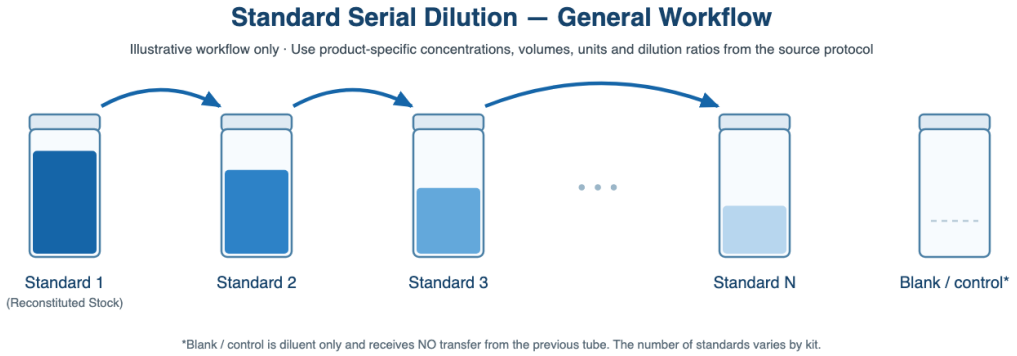
2. If the kit will not be used up in 1 time, please only take out strips and reagents for present experiment, and save the remaining strips and reagents as specified.

3. Dilute the 25× Wash Buffer into 1× Wash Buffer with double-distilled Water.

4. Standard Working Solution - Centrifuge the Standard at 1000 × g for 1 minute. Reconstitute the Standard with 1.0 mL of Standard Diluent Buffer, kept for 10 minutes at room temperature, shake gently (not to foam). The concentration of the Standard in the stock solution is 20 ng/mL. Please prepare 7 tubes containing 0.5 mL Standard Diluent Buffer and use the Diluted Standard to produce a double dilution series according to the picture shown below. To mix each tube thoroughly before the next transfer, pipette the solution up and down several times. Set up 7 points of Diluted Standard such as 20 ng/mL, 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.625 ng/mL, 0.3125 ng/mL, and the last EP tubes with Standard Diluent is the Blank as 0 ng/mL. In order to guarantee the experimental results validity, please use the new Standard Solution for each experiment. When diluting the Standard from high concentration to low concentration, replace the pipette tip for each dilution. Note: the last tube is regarded as a Blank and do not pipette solution into it from the former tube.

5. 1× Biotinylated Antibody and 1× Streptavidin-HRP - Briefly spin or centrifuge the stock Biotinylated Antibody and Streptavidin-HRP before use. Dilute them to the working concentration 100-fold with Biotinylated Antibody Diluent and HRP Diluent, respectively.

6. 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. Lyophilized Standards, Biotinylated Antibody, and Streptavidin-HRP are 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 Standard, Biotinylated Antibody and Streptavidin-HRP Working Solution according to the required amount, and use the corresponding Dilution Solution, cannot be mixed used.

9051 Mira Mesa Blvd, #910094
San Diego, CA 92191
Tel: 760-390-3989

4. Bring all reagents to room temperature (18-25°C) 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°C), gently Mix until crystals are completely dissolved.

5. Prepare to dissolve Standard within 15 minutes before the test. This Standard Working Solution can only be used once. If the dissolved Standard is not used up, please discard it. 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.

6. 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.

7. 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. 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. Determine wells for Diluted Standard, Blank and Sample. Prepare 7 wells for Standard, 1 well for Blank. Add 100 µL each of Standard Working Solution (please refer to Reagent Preparation), or 100 µL of samples into the appropriate wells. Cover with the Plate Cover. Incubate for 80 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.

2. Pour out the liquid of each well. Aspirate the solution and wash with 200 µL of 1× Wash Solution to each well and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper. Notes: (a) When adding Washing Solution, the pipette tip should not touch the wall of the wells to a void contamination. (b) Pay attention to pouring the washing liquid directly to ensure that the washing liquid does not contaminate other wells.

3. Add 100 µL of Biotinylated Antibody Working Solution to each well, cover the wells with the Plate Cover and incubate for 50 minutes at 37°C.

4. Repeat the aspiration, wash process for total 3 times as conducted in step 2.

5. Add 100 µL of Streptavidin-HRP Working Solution to each well, cover the wells with the plate sealer and incubate for 50 minutes at 37°C.

6. Repeat the aspiration, wash process for total 5 times as conducted in step 2.

7. Add 90 µL of TMB Substrate Solution to each well. Cover with a new Plate Cover. Incubate for 20 minutes at 37°C (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.

8. 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.

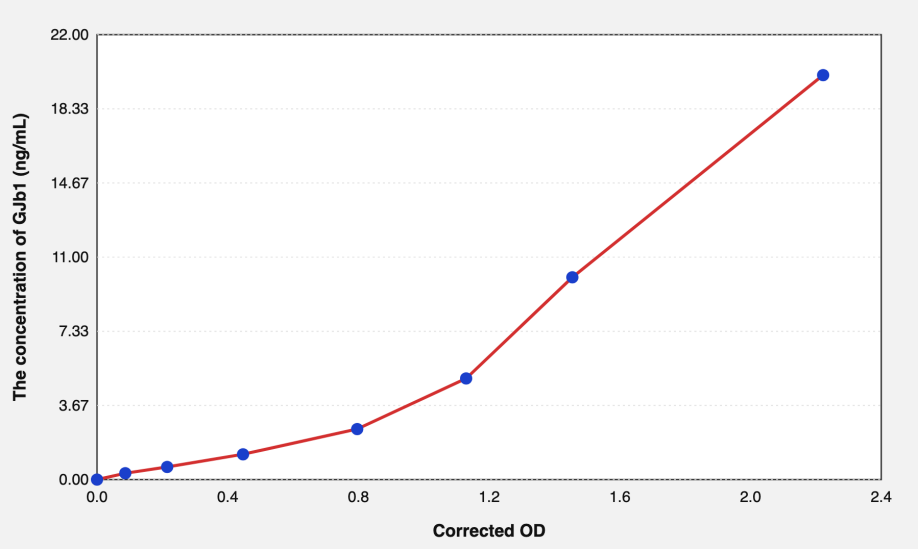
9. Wipe off any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

9051 Mira Mesa Blvd, #910094
San Diego, CA 92191
Tel: 760-390-3989

Calculation of Results

Average the duplicate readings for each Standard, Control, and Samples and subtract the average zero Standard optical density. Construct a Standard curve with the Simian GJb1 concentration on the y-axis and absorbance on the x-axis, and draw a best fit curve through the points on the graph. 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	Corrected OD
20	2.345	2.258
10	1.565	1.478
5	1.235	1.148
2.5	0.896	0.809
1.25	0.541	0.454
0.625	0.305	0.218
0.3125	0.175	0.088
0	0.087	0.000



Note: this graph is for reference only.

Precision

Intra-assay 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

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 Simian GJb1 and the recovery rates were calculated by comparing the measured value to the expected amount of Simian GJb1 in samples.

Matrix	Recovery range	Average
Serum (n = 5)	95-107%	101%
EDTA plasma (n = 5)	85-99%	92%
Heparin plasma (n = 5)	80-92%	86%

Linearity

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of Simian GJb1 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)	97-105%	95-104%	87-96%	92-101%
EDTA plasma (n = 5)	85-93%	82-90%	93-102%	93-106%
Heparin plasma (n = 5)	90-98%	80-93%	92-105%	87-98%

Declaration

1. For research use only. Not intended for diagnostic use.

2. The kit may not be suitable for special experimental samples where the validity of the experiment itself is uncertain, such as gene knockout experiments.

3. 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.

4. 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.

9051 Mira Mesa Blvd, #910094
San Diego, CA 92191
Tel: 760-390-3989

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.
	Biotinylated Antibody 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

9051 Mira Mesa Blvd, #910094
San Diego, CA 92191
Tel: 760-390-3989

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.
Description of results	**Possible reason**	**Recommendations and precautions**
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.

Troubleshooting (continued)		
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.

9051 Mira Mesa Blvd, #910094
San Diego, CA 92191
Tel: 760-390-3989

Troubleshooting (continued)		
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
Description of results	**Possible reason**	**Recommendations and precautions**
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.

9051 Mira Mesa Blvd, #910094
San Diego, CA 92191
Tel: 760-390-3989

Troubleshooting (continued)		
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.