Technical Manual

Human anti-thrombin III antibody ELISA Kit

- **Catalogue Code: HUFI03081**
- **Sandwich ELISA Kit (Double Antigen)**
- **Research Use Only**

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1. Key features and Sample Types

Aliases:

anti-thrombin III antibody ELISA Kit

Uniprot:

P01008

Detection method:

Sandwich, Double Antigen

Sample Type:

Serum, Plasma and other biological fluids

Reactivity:

Human

Range:

1.563-100ng/mL

Sensitivity:

0.938ng/mL

Storage:

Please see Kit contents table

Expiry:

See Kit Label

2. Storage & Expiry

Assay Genie ELISA Kits are shipped on ice packs. Please store this ELISA Kit according to the directions in the kit contents on page 5.

3. Description and Principle

The Assay Genie Sandwich ELISA kit is a highly sensitive assay for the quantitative measurement of a specific analyte in the following samples: serum, blood, plasma, cell culture supernatant and other related supernatants and tissues.

How do our ELISA kits work?

The Assay Genie (enzyme-linked immunosorbent assays) assay kits are designed for the quantitative measurement of analytes in a wide variety of samples. As today's scientists demand premium quality, consistent data, Assay Genie have developed a range of sensitive, fast, and reliable ELISA kit assays to meet and exceed those demands.

This kit was based on sandwich enzyme-linked immune-sorbent assay technology. Antigen was pre-coated onto the 96-well plate. The biotin conjugated antigen was used as the detection antigen. The standards and samples are added to the wells and after incubation, unbound conjugates were removed using wash buffer. Then, biotinylated detection antigen was added to bind with anti-target antibody conjugated to the coated antigen. After washing off unbound conjugates, HRP-Streptavidin was added. After a third washing, TMB substrates were added to visualize HRP enzymatic reaction. TMB was catalysed by HRP to produce a blue coloured product that turned yellow after adding acidic stop solution. Read the O.D. absorbance at 450nm in a microplate reader. The concentration of target antigen in the sample is positively correlated with OD450 and can be calculated by plotting the standard curve.

4. Kit Contents

Each kit contains reagents for either 48 or 96 assays, please store the reagents per conditions below.

No.	Component	48-Well Kit	96-Well Kit	Storage
1	ELISA Microplate (dismountable)	8 x 6	8 x 12	Place the test strips into a sealed foil bag with the desiccant. Store for 1 month at 2-8°C; Store for 6 months at -20°C.
2	Lyophilized Standard	1 vial	2 vials	Place the standards into a sealed foil bag with the desiccant. Store for 1 month at 2-8°C; Store for 6 months at -20°C.
3	Biotin-labelled Antigen (Concentrated, 100X)	60 µL	120 µL	2-8°C (Avoid Direct Light)
4	HRP-Streptavidin Conjugate (SABC, 100X)	60 µL	120 µL	
5	TMB Substrate	5 mL	10 mL	
6	Sample Dilution Buffer	10ml	20ml	2-8°C
7	Antigen Dilution Buffer	5 mL	10 mL	
8	SABC Dilution Buffer	5 mL	10 mL	
9	Stop Solution	5ml	10ml	
10	Wash Buffer (25x)	15 mL	30 mL	
11	Plate Sealer	3 pieces	5 pieces	
12	Product Description	1	1	

Please note that the liquid bottles contain slightly more reagent than indicated on the label. Please use a pipette to accurately measure out required amounts.

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.

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 plates are kept dry to avoid contamination.

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

10. Please wear the lab coat, mask, and gloves to protect yourself during the assay. Especially, for the detection of blood or other body fluid samples. Please follow regulations on safety protection of biological laboratory.

Quick Protocol

Step 1: Add 100ul standard or sample into each well, seal the plate and incubate for 90 minutes at 37°C.

Washing: Wash the plate twice.

Step 2: Add 100ul biotin-labelled antigen working solution into each well, seal the plate and incubate for 60 minutes at 37°C.

Washing: Wash the plate three times.

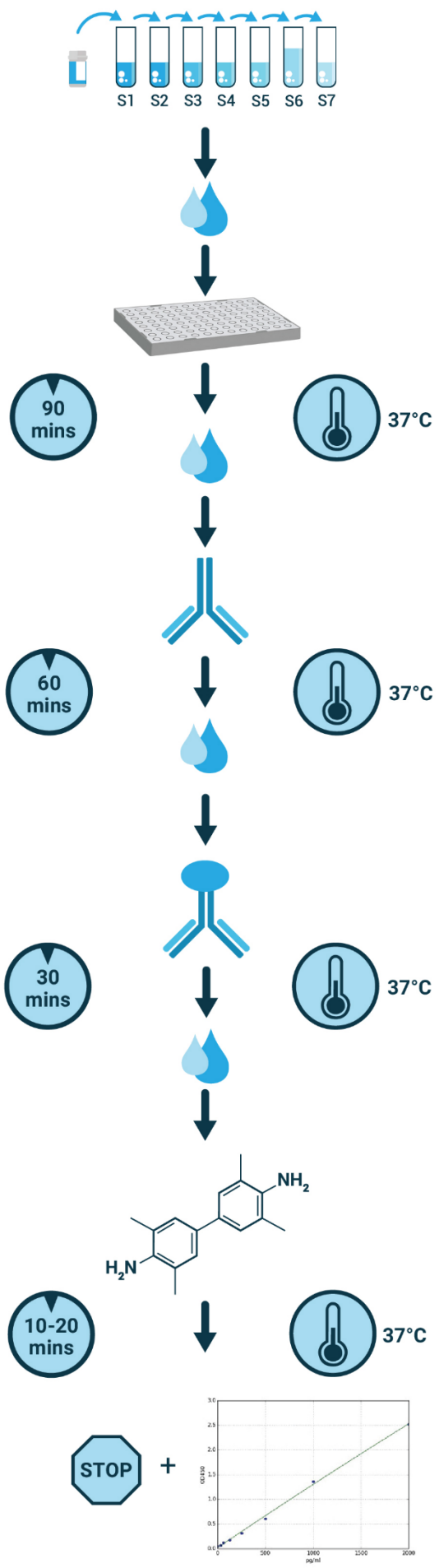
Step 3: Add 100ul SABC working solution into each well, seal the plate and static incubate for 30 minutes at 37°C.

Washing: Wash the plate five times.

Step 4: Add 90ul TMB substrate solution, seal the plate and incubate for 10-20 minutes at 37°C. (Accurate TMB visualization control is required.)

Step 5: Add 50ul stop solution. Read at 450nm immediately and calculate.

5. Workflow Overview



Prepare plate standards and reagents.

Add 100µl of samples, standards, and controls.

Wash plate x 2

Add 100µl Biotin-labelled Antigen working solution.

Wash plate x 3

Add 100µl SABC working solution.

Wash plate x 5

Add 90µl TMB Substrate

Add 50µl STOP solution and read absorbance @ 450nm.

6. Sample Preparation

Notes for Samples:

- Blood collection tubes should be disposable and endotoxin free. Avoid using haemolyzed and lipemic samples.
- Samples must be stored for less than 5 days at 2-8°C; for 6 months at -20°C; and 2 years at -80°C. Store samples in liquid nitrogen for a longer storage. When melting frozen samples, a quick incubation in a water bath at 15 -25°C can reduce the effect of ice crystals on the samples. After melting, centrifuge to remove any precipitate, and then mix well.
- The detection range of this kit is not equivalent to the concentration of analytes in samples. For analyses with higher or lower concentrations, please properly dilute or concentrate your samples.
- A pre-test is recommended for special samples without reference data to validate their validity.
- Recombinant protein may not match with the capture or detection antigen in this kit and may result in a failed assay.

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: Place your whole blood sample at room temperature for 2 hours or at 2-8°C overnight. Centrifuge for 20min at 1,000xg and collect the supernatant to detect immediately. Or you can aliquot the supernatant and store it at -20°C or -80°C for future assays.

Plasma: EDTA-Na2/K2 is recommended as the anti-coagulant for this kit. Centrifuge samples for 15 minutes at 1,000×g at 2-8°C within 30 minutes of sample collection. Once you collect the supernatant you can detect immediately. Alternatively, you can aliquot the supernatant and store it at -20°C or -80°C for future assays. For other anti-coagulant types, please refer to the sample preparation guideline.

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

Cell culture supernatant: Collect the cell culture media by pipette, followed by centrifugation at 4°C for 20 mins at 1,000 x g. Collect the clear supernatant and assay immediately. Or you can aliquot the supernatant and store it at -80°C for future assays.

Cell lysates:

Suspension Cell Lysate: Centrifuge at your sample at 2,500 rpm for 5 minutes (2-8°C). Add pre-cooled sterile PBS and gently mix. Re-centrifuge your sample as above to collect your cell pellet. Discard PBS following centrifugation, while not disrupting your pellet. Add 0.5-1 ml cell lysis buffer *(NP-40 lysis buffer or Triton X-100 surfactant are not recommended due to their interference with the antigen-antibody reaction)*. Add a suitable protease inhibitor (e.g. PMSF, working concentration: 1 mmol/L). Lyse cells on ice for 30 min - 1 hour. During the lysis process, use a pipette tip or intermittent shaking of the centrifuge tube to completely lyse the cells. Alternatively, subject cells to fragmentation by sonication (14 μM for 30 s) or ultrasonic cell disruptor (300 W, 3~5 s/time. 30 s intervals, four-five times). At the end of the lysis or ultrasonic disruption steps, centrifuge at your sample at 10,000 rpm for 10 minutes (2-8°C). Add the supernatant into a fresh Eppendorf tube and assay immediately, alternatively store at -80°C.

Adherent Cell Lysate: Aspirate your supernatant, followed by washing your cells with pre-cooled sterile PBS three times. Add 0.5-1ml cell lysis buffer and the appropriate protease inhibitor (e.g. PMSF, working concentration: 1mmol/L). Scrape the adherent cells with a cell scraper and transfer the sample to a centrifuge tube. Allow the cells to lyse while incubating on ice for 30 min to 1 hour. Alternatively disrupt the cells via sonication as detailed above.

Following cell lysis, centrifuge your samples at 10,000 rpm at 2-8°C for 10 minutes. Then, the supernatant is added into Eppendorf tube and detect immediately. Or you can aliquot the supernatant and store it at - 80°C for future assays.

Tissue Homogenates: Rinse the tissue with 1X Sterile PBS to remove excess blood. Mince tissue after weighing it and homogenize in sterile PBS (the volume depends on the weight of the tissue. 9mL PBS (including protease inhibitors) would be appropriate for 1 gram of tissue). To further lyse your 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 minutes at 5,000 x g and the supernatant is removed for assaying.

Optimal protein concentration for ELISA assays should be within 1-3mg/ml. Some tissue samples such as liver, kidney and pancreas contain a higher endogenous peroxidase

concentration and may react with TMB substrate causing false positivity. In that case, try to use 1% H_2O_2 for 15min inactivation and perform the assay again.

Notes: PBS buffer or the mild RIPA lysis (pH 7.3) can be used as lysis. Avoid using any reagents containing NP-40 lysis buffer, Triton X-100 surfactant, or DTT due to their effects on the kit's performance. We recommend using 50mM Tris, 0.9%NaCL, 0.1%SDS at pH7.3.

Other Biological Samples:

Centrifuge samples for 15 minutes at 1000×g at 2-8°C. Collect the supernatant to detect immediately. Or you can aliquot the supernatant and store it at -80°C for future's assay.

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 350µl 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 350µl 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 (18 - 25°C). For repeated assays, please use only strips and standards required and store remaining reagents at the appropriate temperatures.

1. Wash Buffer:

Dilute 30 ml (15 ml for 48T) of Concentrated Wash Buffer into 750 ml (375 ml for 48T) of Wash Buffer with deionized or distilled water. Store unused solution at 4°C. If crystals have formed in the concentrate, warm at 40°C in water bath (Heating temperature should not exceed 50°C) and mix gently until crystals have completely dissolved. The solution should be cooled to room temperature before use.

2. Standard Dilution:

1) Centrifuge the standard tube for 1 min at 10,000 x g.

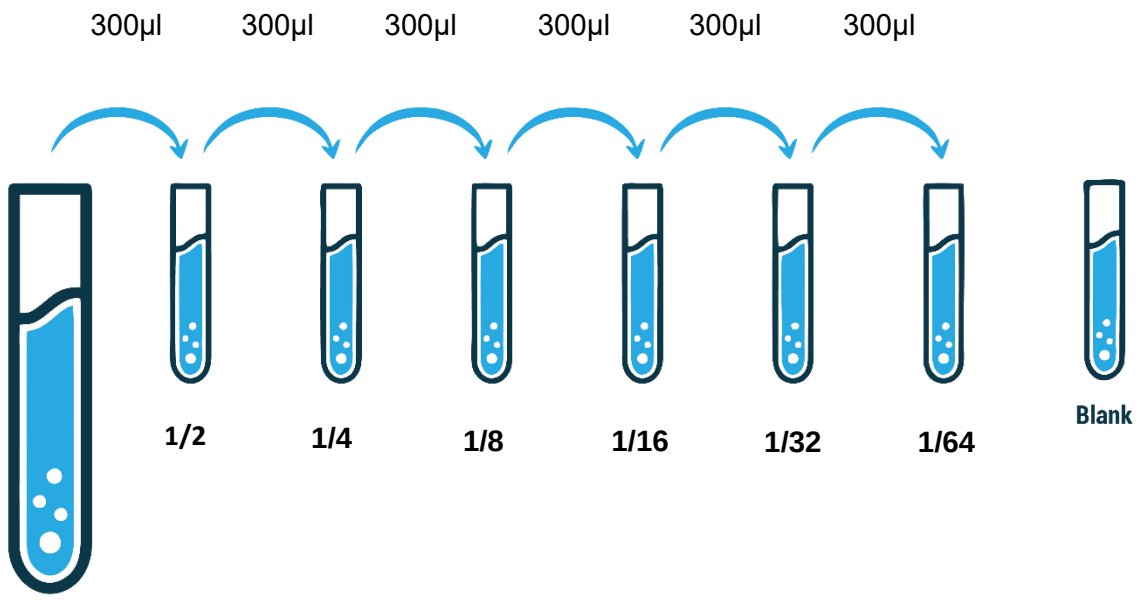
2). Add 1ml of Sample dilution buffer into one Standard tube (labelled as Stock Solution), keep the tube at room temperature for 2 min. Invert the tube several times to mix (or use a low-speed vortex mixer for 3 – 5 seconds).

3) Finally, centrifuge for 1 min at 1,000 x g to collect liquid at the bottom of the tube and remove bubbles.

Note: If the standard vial concentration is different to the highest value in the range (please see page 3), please dilute using sample buffer to match highest range value to create stock solution.

2). Label 7 Eppendorf tubes with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank respectively. Aliquot 300µl of the Sample dilution buffer into each tube. Add 300µl of the above (Stock Solution) standard solution into 1st tube and mix thoroughly. Transfer 300µl from 1st tube to 2nd tube and mix thoroughly. Transfer 300µl from 2nd tube to 3rd tube and mix thoroughly, and so on. Sample dilution buffer is used as blank control.

DILUTION SERIES



Note: The standard solutions are best used within 2 hours. The standard solution series should be kept at 4°C for up to 12 hours. Or store at -20 °C for up to 48 hours. Avoid repeated freeze-thaw cycles.

3. Preparation of biotin-labelled Antigen working solution:

The antigen working solution should be prepared within 30 minutes of starting the assay and it cannot be stored for a long period of time.

1. Calculate the total volume of the working solution: 100µl / well × quantity of wells (Allow 100-200µl more than the total volume).
2. Centrifuge the biotin-labelled antigen for 1 min at 1,000 x g to collect all the liquid at the bottom of the tube.
3. Dilute the biotin-detection antigen with antigen dilution buffer at 1:100 and mix thoroughly (i.e. Add 1µl of Biotin-labelled Antigen into 99µl of Antigen Dilution Buffer.)

4. Preparation of HRP-Streptavidin Conjugate (SABC) working solution:

The SABC working solution should be prepared within 30 minutes of starting the assay and it cannot be stored for a long period of time.

1. Calculate the total volume of the working solution: 100µl / well × quantity of wells. (Allow 100-200µl more than the total volume).
2. Centrifuge the concentrated SABC solution for 1 min at 1,000 x g to collect all the liquid at the bottom of the tube.
3. Dilute the SABC with SABC dilution buffer at 1:100 and mix thoroughly. (i.e. Add 1µl of SABC into 99µl of SABC dilution buffer.)

8. Assay Procedure

Before adding to the wells, equilibrate the TMB substrate for at least 30 mins at room temperature. When diluting samples and reagents, ensure they are mixed completely and evenly. It is recommended to plot a standard curve for each test.

1. Set standard, **test sample (diluted at least ½ with Sample Dilution Buffer)** and control (zero) wells on the pre-coated plate and record their positions. It is recommended to measure each standard and sample in duplicate. ☐
2. **Standards:** Aliquot 100µl of standard solutions into the standard wells. ☐
3. **Dilution Buffer:** Add 100µl of Sample dilution buffer into the control (blank) well. ☐
4. **Samples:** Add 100µl of properly diluted sample into test sample wells. ☐
5. **Incubate:** Seal the plate with a cover and incubate at 37 °C for 90 mins. ☐

6. **Wash:** Remove the cover, aspirate the liquid from the plate and wash plate 2 times with Wash Buffer. **Do NOT let the wells dry completely at any time.** ☐
7. **Biotin-labelled Antigen:** Add 100µl of Biotin-labelled Antigen working solution to the bottom of each well (standard, test sample & zero wells) without touching the side walls. ☐
8. **Incubate:** Seal the plate with a cover and incubate at 37°C for 60 mins. ☐
9. **Wash:** Remove the cover, and wash plate 3 times with Wash buffer. **Let the Wash Buffer stay in the wells for 1-2 minutes for each wash.** ☐
10. **HRP-Streptavidin Conjugate (SABC):** Add 100µl of SABC working solution into each well, cover the plate and incubate at 37°C for 30 mins. ☐
11. **Wash:** Remove the cover and wash plate 5 times with Wash buffer. **Let the Wash Buffer stay in the wells for 1-2 minutes for each wash.** ☐
12. **TMB Substrate:** Add 90 µl of TMB substrate into each well, cover the plate and incubate at 37°C in dark for 10-20 mins. (Note: This incubation time is for reference only, the optimal time should be determined by the end-user.) As soon as a blue colour develops in the first 3-4 wells (with most concentrated standards) and the other wells show no obvious colour, terminate the reaction by moving to Step 13. ☐
13. **Stop Solution:** Add 50µl of Stop solution into each well and mix thoroughly. The colour changes into yellow immediately. ☐
14. **OD Measurement:** Read the O.D. absorbance at 450 nm in a microplate reader immediately after adding the stop solution. ☐

9. Data Analysis

Calculate the mean OD450 value of the duplicate readings for each standard, control, and sample. Then, obtain the corrected OD450 by subtracting the OD450 blank.

Create a four-parameter logistic curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis. (Remove the OD450 blank during plotting.) Alternatively, you can use curve fitting software.

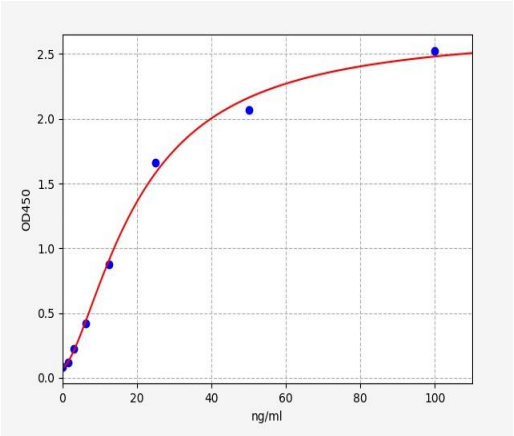
Calculate the sample concentration by substituting OD450 value into the standard curve. Diluted samples should be multiplied by the relevant dilution ratio.

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.



Specificity

This assay has high sensitivity and excellent specificity for detection of anti-thrombin III antibody. No significant cross-reactivity or interference between anti-thrombin III antibody and analogues was observed.

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

Recovery

Matrices listed below were spiked with a certain level of anti-thrombin III antibody and the recovery rates were calculated by comparing the measured value to the expected amount of anti-thrombin III antibody in the samples.

Matrix (N=5)	Recovery Range (%)	Average (%)
Serum	89-104	99
EDTA Plasma	95-102	98
Heparin Plasma	86-100	96

Linearity

The linearity of the kit was assayed by testing the samples spiked with appropriate concentration of anti-thrombin III antibody and their serial dilutions.

Sample (N=5)	1:2	1:4	1:8
Serum	85-104%	92-104%	94-105%
EDTA Plasma	83-98%	83-93%	84-95%
Heparin Plasma	83-98%	88-100%	89-100%

Precision

- **Intra-Assay:** CV<8%
- **Inter-Assay:** CV<10%

Stability

The stability of the anti-thrombin III antibody is determined by the loss rate of activity. The loss rate of this kit is less than 10% within the expiration date under appropriate storage conditions.

Standard (n=5)	37°C for 1 month	4°C for 6 months
Average (%)	80	95-100

To minimize extra influence on the performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.

ELISA Troubleshooting

If the ELISA result is unsatisfied, please take a screenshot for the staining result and store the OD data

Problem	Possible Causes	Solutions
Standard curve without signal	Incorrect order for adding reagents	Confirm the required reagent added in each step. Also repeat the assay and verify.
	Components used from different kits	Use the component included in the same kit. Also repeat the assay and verify.
	Forget to add some reagents	Verify whether the required reagent is added.
Overflow OD	Use components from different kits, or prepare the working solution with higher concentration	Use the component included in the same kit. Also repeat the assay and verify.
Poor standard curve	Inappropriate curve fitting model	Try to plot the curve by different fitting models.
Samples without signal	The amount of sample is lower than the detection range.	Decrease dilution ratio or concentrate the sample.
	The detection target is incompatible with the buffer.	Verify the compatibility of sample storage buffer with the sample.
	Incorrect preparation of sample	Please refer to sample preparation guideline.
	Longer storage of sample or freeze- thaw cycle	Aliquot and store samples according to the assay requirement.
High CV%	Precipitate is formed in the well during staining.	Increase the dilution ratio of the sample.
	Unclean plate	Don't touch the bottom of the plate during the assay.
	Foam is found in the well.	Avoid foaming during reading in a microplate reader.
	Each well is washed unevenly.	Check whether the tube of the washer is smooth.
	Reagents are not completely mixed.	Mix all reagents completely.
	Inconsistent pipetting	Use calibrated pipette and correct pipetting method.
Standard curve with low signal	Standards are improperly reconstituted.	Before opening, shortly centrifuge the lyophilized standard tube till complete dissolution.
	Standards have been degraded.	Follow suggested storage conditions for

Notes: