Version 03-09/25

User's Manual

Mouse Anti-tissue transglutaminase IgA ELISA Kit

REF **DEIA-BJ2623**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

For quantitative detection of TGA-IgA in serum, plasma, tissue homogenates and other biological fluids.

Principles of Testing

This kit was based on indirect enzyme-linked immune-sorbent assay technology. Antigen was pre-coated onto 96-well plates. And the biotin conjugated antibody was used as detection antibodies. The standards, test samples and biotin conjugated detection antibody were added to the wells subsequently, and washed with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the target amount of sample captured in plate. Read the O.D. absorbance at 450nm in a microplate reader, and then the concentration of target can be calculated.

Reagents And Materials Provided

1. ELISA Microplate: (Dismountable) 8×12, 2-8°C/-20°C
2. Lyophilized Standard: 2 vial, 2-8°C/-20°C
3. Biotin-labeled Antibody (Concentrated, 100X): 1 vial, 120 µl, 2-8°C (Avoid Direct Light)
4. HRP-Streptavidin Conjugate (SABC, 100X): 120 µl, 2-8°C (Avoid Direct Light)
5. TMB Substrate: 10 ml, 2-8°C (Avoid Direct Light)
6. Sample Dilution Buffer: 20 ml, 2-8°C
7. Antibody Dilution Buffer: 10 ml 2-8°C
8. SABC Dilution Buffer: 10 ml, 2-8°C
9. Stop Solution: 10 ml, 2-8°C
10. Wash Buffer(25X): 30 ml, 2-8°C
11. Plate Sealer: 5 pieces
12. Product Description: 1 copy

Materials Required But Not Supplied

1. Microplate reader (wavelength: 450 nm)
2. 37°C incubator (CO_2 incubator for cell culture is not recommended.)
3. Automated plate washer or multi-channel pipette/5ml pipettor (for manual washing purpose)
4. Precision single (0.5-10 µL, 5-50 µL, 20-200 µL, 200-1000 µL) and multi-channel pipette with disposable tips(calibration is required before use.)
5. Sterile tubes and Eppendorf tubes with disposable tips
6. Absorbent paper and loading slot

7. Deionized or distilled water

Storage

2-8°C

Specimen Collection And Preparation

1. **Serum**

Place whole blood sample at room temperature for 2 hours or at 2-8°C overnight. Centrifuge for 20min at 1000xg and collect the supernatant to detect immediately. Or you can aliquot the supernatant and store it at -20°C or -80°C for future's assay.

2. **Plasma**

EDTA-Na2/K2 is recommended as the anticoagulant. Centrifuge samples for 15 minutes at 1000 ×g 2-8°C within 30 minutes after collection. Collect the supernatant to detect immediately. Or you can aliquot the supernatant and store it at -20°C or -80°C for future's assay. For other anticoagulant types and uses, please refer to the sample preparation guideline.

3. **Tissue Sample**

Generally tissue samples are required to be made into homogenization. Protocol is as below:

3. 1. Place the target tissue on the ice. Remove residual blood by washing tissue with pre-cooling PBS buffer (0.01 M, pH = 7.4). Then weigh for usage.
3. 2. Use lysate to grind tissue homogenates on the ice. The adding volume of lysate depends on the weight of the tissue. Usually, 9 mL PBS would be appropriate to 1 gram tissue pieces. Some protease inhibitors are recommended to add into the PBS (e.g. 1 mM PMSF).
3. Do further process using ultrasonic disruption or freeze-thaw cycles (Ice bath for cooling is required during ultrasonic disruption; Freeze-thaw cycles can be repeated twice.) to get the homogenates.
3. 4. Homogenates are then centrifuged for 5 minutes at 5000 ×g. Collect the supernatant to detect immediately. Or you can aliquot the supernatant and store it at -20°C or -80°C for future's assay.
3. 5. Determine total protein concentration by BCA kit for further data analysis. Usually, total protein concentration for Elisa assay should be within 1-3mg/ml. Some tissue samples such as liver, kidney, pancreas which containing a higher endogenous peroxidase concentration may react with TMB substrate causing false positivity. In that case, try to use 1% H2O2 for 15min inactivation and perform the assay again.

Notes: PBS buffer or the mild RIPA lysis can be used as lysates. While using RIPA lysis, make the PH=7.3. Avoid using any reagents containing NP-40 lysis buffer, Triton X-100 surfactant, or DTT due to their severe inhibition for kits' working. We recommend using 50 mM Tris + 0.9% NaCL+0.1% SDS, PH7.3. You can prepare by yourself or contact us for purchasing.

4. **Cell Culture Supernatant**

Collect the supernatant: Centrifuge at 2500 rpm at 2-8°C for 5 minutes, then collect clarified cell culture supernatant to detect immediately. Or you can aliquot the supernatant and store it at -80°C for future's assay.

5. **Cell Lysate**

5. 1. Suspension Cell Lysate: Centrifuge at 2500 rpm at 2-8°C for 5 minutes and collect cells. Then add pre-

cooling PBS into collected cell and mix gently. Recollect cell by repeating centrifugation. Add 0.5-1ml cell lysate and appropriate protease inhibitor (e.g. PMSF, working concentration: 1mmol/L). Lyse the cell on ice for 30min-1h or disrupt the cell by ultrasonic disruption.

5. 2. Adherent Cell Lysate: Absorb supernatant and add pre-cooling PBS to wash three times. Add 0.5-1ml cell lysate and appropriate protease inhibitor (e.g. PMSF, working concentration: 1mmol/L). Scrape the adherent cell with cell scraper. Lyse the cell suspension added in the centrifuge tube on ice for 30min-1h or disrupt the cell by ultrasonic disruption.
5. 3. During lysate process, use the tip for pipetting or intermittently shake the centrifugal tube to completely lyse the protein. Mucilaginous product is DNA which can be disrupted by ultrasonic cell disruptor on ice. (3~5mm probe, 150-300W, 3~5 s/time, 30s intervals for 1~2s working).
5. 4. At the end of lysate or ultrasonic disruption, centrifuge at 10000rpm at 2-8°C for 10 minutes. Then, the supernatant is added into EP tube to detect immediately. Or you can aliquot the supernatant and store it at -80°C for future's assay.

Notes: Read notes in tissue sample.

6. **Other Biological Sample**

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.

Notes for Samples

1. Blood collection tubes should be disposable and non-endotoxin. Avoid to use hemolyzed and lipemia samples.
2. The best sample storage condition: less than 5 days at 2-8°C; within 6 months at -20°C; within 2 years at -80°C. Stored in liquid nitrogen for a longer storage. When melting frozen samples, rapid water bath at 15-25°C can decrease the effect of ice crystal (0°C) on the sample. After melting, centrifuge to remove the precipitate, and then mix well.
3. The detection range of this kit is not equivalent to the concentration of analyze in the sample. For analyses with higher or lower concentration, please properly dilute or concentrate the sample.
4. Pretest is recommended for special samples without reference data to validate the validity.
5. Recombinant protein may not match with the capture or detection antibody in the kit, resulting in the undetectable assay.

The user should estimate the concentration of target protein in the test sample, and select a proper dilution factor to make the diluted target protein concentration fall in the optimal detection range of the kit. Dilute the sample with the provided dilution buffer, and several trials may be necessary. The test sample must be well mixed with the dilution buffer. And also standard curves and sample should be making in pre-experiment. If samples with very high concentrations, dilute samples with PBS first and then dilute the samples with Sample Dilution.

The matrix components in the sample will affect the test results, which it need to be diluted at least 1/100 with Sample Dilution Buffer before testing!

Reagent Preparation

Take the Elisa kit from the fridge around 20 minutes earlier and equilibrate to room temperature(18-25°C). For repeated assays, please just take the strips and standards required for the current assay, store the rest materials according to the relevant condition.

1. **Wash Buffer**

Dilute 30 ml concentrated wash buffer to 750 ml wash buffer with deionized or distilled water and mix well. (The recommended resistivity of ultrapure water is 18 MΩ.) Alternatively, take appropriate amount of concentrated wash buffer according to the assay requirement, then create a 25-fold dilution and mix well. Store the rest solution at 2-8°C.

Crystals formed in the concentrated wash buffer can be heated by water bath at 40°C till complete dissolution. (Heating temperature should be below 50°C.) Mix well for the next step. It's better to use up the prepared wash buffer in one day. Store the rest buffer at 2-8°C within 48h.

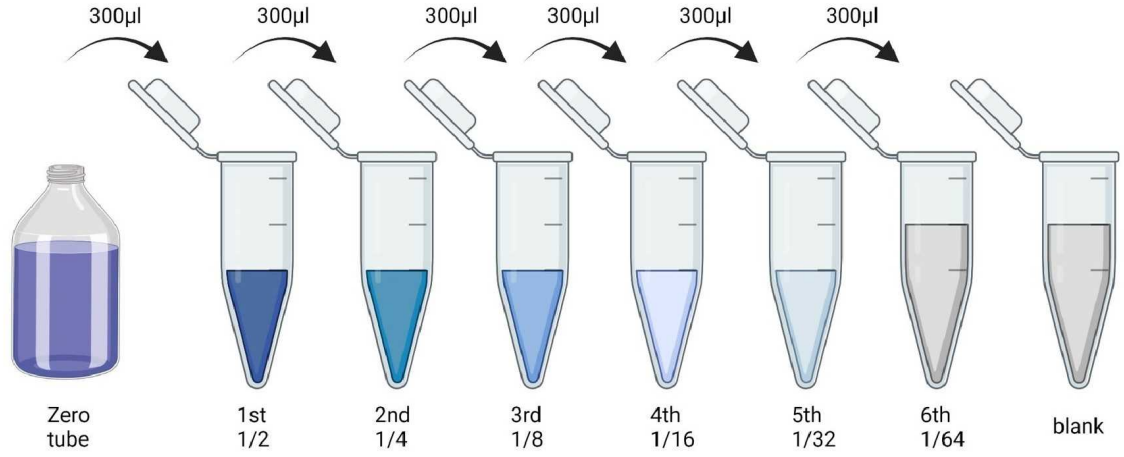
2. **Standards**

2. 1. Centrifuge standards tube for 1min at 10000 xg. Label it as Zero tube.

2. Add 1ml sample dilution buffer into the standard tube. Tighten the tube cap and Let it stand for 2 min at room temperature. Invert the tube several times to mix gently. (Or you can mix it using a low speed vortex mixer for 3-5 seconds.)

2. 3. Centrifuge the tubes for 1min at 1000 xg, making the liquid towards the bottom of tube and removing possible bubbles.

2. 4. Standard dilution: Label 7 EP tubes with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank respectively. Add 0.3 ml of the sample dilution buffer into each tube. Add 0.3ml solution from zero tube into 1/2 tube and mix them thoroughly. Transfer 0.3 ml from 1/2 tube into 1/4 tube and mix them thoroughly. Transfer 0.3 ml from 1/4 tube into 1/8 tube and mix them thoroughly, so on till 1/64 tube. Now blank tube only contain 0.3 ml sample dilution buffer.



Notes: Store the zero tube with dissolved standards at 2-8°C and use it within 12h. Other diluted working solutions containing standards should be used in 2h.

3. **Preparation of Biotin-labeled Antibody Working Solution**

The working solution should be prepared within 30 min before the assay and can't be stored for a long time.

3. 1. Calculate required total volume of the working solution: 100 ul/well x quantity of wells. (It's better to prepare additional 100 ul-200 ul.)

3. 2. Centrifuge for 1min at 1000 xg in low speed and bring down the concentrated biotin-labeled antibody to the bottom of tube.

3. Dilute the biotinylated detection antibody with antibody dilution buffer at 1:99 and mix them thoroughly. (e.g.

Add 10 ul concentrated biotin-labeled antibody into 990 ul antibody dilution buffer.)

4. **Preparation of HRP-Streptavidin Conjugate (SABC) Working Solution**

The working solution should be prepared within 30 min before the assay and can't be stored for a long time.

4. 1. Calculate required total volume of the working solution: 100 ul/well x quantity of wells. (It's better to prepare additional 100ul-200ul.)
4. 2. Centrifuge for 1min at 1000 xg in low speed and bring down the concentrated SABC to the bottom of tube.
4. 3. Dilute the concentrated SABC with SABC dilution buffer at 1:99 and mix them thoroughly. (e.g. Add 10 ul concentrated SABC into 990 ul SABC dilution buffer.)

Assay Procedure

When diluting samples and reagents, they must be mixed completely. It's recommended to plot a standard curve for each test.

1. Set standard, pilot samples, control (blank) wells on the pre-coated plate respectively, and then, records their positions. It's recommended to measure each standard and sample in duplicate to decrease experimental errors.
2. Standards and samples loading: Aliquot 100ul of zero tube, 1st tube, 2nd tube, 3rd tube, 4th tube into each standard well. Also add 100ul sample dilution buffer into the control (blank) well. Then, add 100ul pilot samples into each sample well. Seal the plate and static incubate for 90 minutes at 37°C. (Add the solution to the bottom of each well. Mix gently and without touch the sidewall and foam the sample.)
3. Wash twice: Remove the cover, then absorb the liquid in the plate or tap the plate on a clean absorbent paper two or three times. Add 350ul wash buffer into each well without immersion. Discard the liquid in the well and tap on the absorbent paper again. Repeat the washing step twice.
4. Biotin-labeled Antibody: Add 100ul biotin-labeled antibody working solution into each well. Seal the plate and static incubate for 60 minutes at 37°C.
5. Wash three times: Remove the cover, then absorb the liquid in the plate or tap the plate on a clean absorbent paper two or three times. Add 350ul wash buffer into each well and immerse for 1min. Discard the liquid in the well and tap on the absorbent paper again. Repeat the washing step three times.
6. HRP-Streptavidin Conjugate (SABC): Add 100ul SABC working solution into each well. Seal the plate and static incubate for 30 minutes at 37°C. (Put the whole bottle of TMB into the 37°C incubator to equilibrate for 30min.)
7. Wash five times: Remove the cover, and then wash the plate with wash buffer five times. Read washing method in step 5.
8. TMB Substrate: Add 90ul TMB Substrate into each well, seal the plate and static incubate at 37°C in dark within 10-20 minutes. Run the microplate reader and preheat for 15min.

(Notes: Please do not use the reagent reservoirs used by HRP couplings. The reaction time can be shortened or extended according to the actual color change, but not more than 30 minutes. You can terminate the reaction when apparent gradient appeared in standard wells. Weaker or stronger color intensity is unacceptable.)

9. Stop: Keep the liquid in the well after staining. Add 50ul stop solution into each well. The color will turn yellow immediately. The order for adding stop solution and TMB substrate solution is the same.

10. OD Measurement: Read the O.D. absorbance at 450nm in a microplate reader immediately. (If your microplate reader has a choice of correction wavelength, set it to 570nm or 630nm. Correct the read value to the OD450 value minus the OD570 or OD630 value. In this way, the OD value of non-chromogenic substances can be corrected and removed, thus obtaining more accurate results. If the microplate reader does not have a 570nm or 630nm wavelength, the original OD450 value can be used.)

Assay Procedure Summary

Set standard, test samples, control (blank) wells on the pre-coated plate respectively, and then, records their positions.

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

Washing: Wash the plate twice without immersion.

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

Washing: Wash the plate three times and immerse for 1min each time.

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

Washing: Wash the plate five times and immerse for 1min each time.

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

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

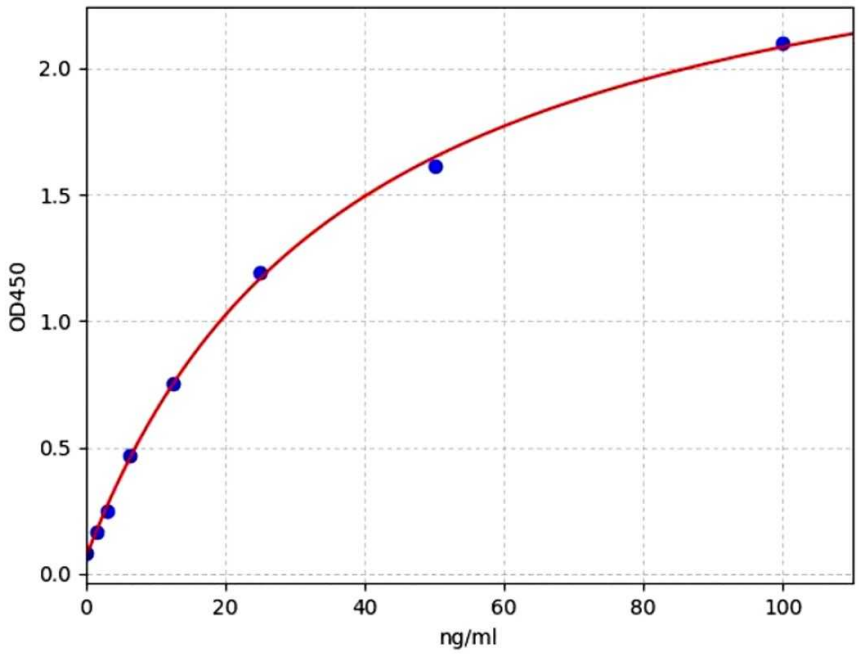
Calculation

1. Calculate the mean OD450 value (using the original OD450 value or the corrected OD450 value) of the duplicate readings for each standard, control, and sample. Then, obtain the value of calculation by subtracting the OD450 blank.
2. 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 the curve fitting software offered by the microplate reader.
3. Calculate the sample concentration by substituting OD450 value into the standard curve. Diluted samples should be multiplied by the relevant dilution ratio.

Typical Standard Curve

Results of a typical standard operation of a TGA-IgA ELISA Kit are listed below. This standard curve was generated at our lab for demonstration purpose only. Users shall obtain standard curve as per experiment by themselves. (N/A=not applicable)

STD.(ng/ml)	OD-1	OD-2	Average	Corrected
0	0.078	0.08	0.079	0
1.562	0.163	0.167	0.165	0.086
3.125	0.241	0.249	0.245	0.166
6.25	0.461	0.475	0.468	0.389
12.5	0.74	0.762	0.751	0.672
25	1.178	1.212	1.195	1.116
50	1.59	1.636	1.613	1.534
100	2.069	2.129	2.099	2.02



Detection Range

1.563-100 ng/ml

Sensitivity

0.938 ng/ml

Specificity

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

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

Linearity

Dilute the sample with a certain amount of TGA-IgA at 1:2, 1:4 and 1:8 to get the recovery range.

Sample	1:2	1:4	1:8
Serum(n=5)	90-105%	90-101%	89-100%
EDTA Plasma(n=5)	86-101%	88-101%	82-98%
Heparin Plasma(n=5)	80-98%	88-97%	88-97%

Recovery

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

Matrix	Recovery Range (%)	Average (%)
Serum(n=5)	89-104	96
EDTA Plasma(n=5)	92-103	96
Heparin Plasma(n=5)	93-101	96

Precautions

1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, pilot experiment using standards and a small number of samples is recommended.
2. After opening and before using, keep plate dry.
3. Before using the kit, spin tubes and bring down all components to the bottom of tubes.
4. Storage TMB reagents avoid light.
5. Washing process is very important, not fully wash easily cause a false positive and high background.
6. Duplicate well assay is recommended for both standard and sample testing.
7. Don't let microplate dry at the assay, for dry plate will inactivate active components on plate.
8. Don't reuse tips and tubes to avoid cross contamination.
9. Please do not mix the reagents in different kits of our company. Do not mix reagents from other manufacturers.
10. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.