Zebrafish Tumor Necrosis Factor Alpha ELISA KIT

BIOENZY
Rev.04-1

Catalog no	**BZ-22272158-EB**
Size	**96 wells**

User Instruction

Detection Range	3.13-200 pg/mL
Sensitivity	1.26 pg/mL
Repeatability	Intra-Assay: CV < 8%; Inter-Assay: CV < 10%
Storage	Keep the **whole kit** before use in: • -20°C for 12 months validity • 2-8°C for 6 months validity Avoid repeated freeze–thaw cycles. Do not use the kit beyond its expiration date.

Specificity

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

Assay 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 Zebrafish TNFa. Standards or samples are added to the appropriate microtiter plate wells then with a biotin-conjugated antibody specific to Zebrafish TNFa. 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 Zebrafish TNFa, 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 Zebrafish TNFa in the samples is then determined by comparing the OD of the samples to the standard curve.

Storage Directions and Kit Components

Keep the **whole kit** before use in:

- -20°C for 12 months validity
- 2-8°C for 6 months validity

After opening handling, store the items separately according to the following directions:

Components	96 Wells	48 Wells	Storage Directions After Opening Handling
Pre-Coated Microplate	12 strips x 8 wells	6 strips x 8 wells	2-8°C (1 week) / -20°C (6 months)
Standard (Lyophilized)	2 vials	1 vial	
Biotinylated Antibody (100×)	120 μL	60 μL	
Streptavidin-HRP (100×)	120 μL	60 μL	
Standard/Sample Diluent Buffer	20 mL	10 mL	2-8°C (Please use it within 6 months after opening)
Biotinylated Antibody Diluent	12 mL	6 mL	
HRP Diluent	12 mL	6 mL	
Stop Reagent	6 mL	3 mL	
Wash Buffer (25×)	20 mL	10 mL	
TMB Substrate Solution	10 mL	6 mL	2-8°C (Please use it within 6 months after opening) (light sensitive, avoid light)
Plate Covers	2 pieces	1 piece	RT
Instruction manual	1	1	

Notes:

- If the whole kit is stored at -20°C, place the kit at 2-8°C the day before the experiment.
- After receiving the kit, the plates can be disassembled to single strips. Please use it in batches on demands.

Material Required But Not Supplied

- Electro-heating standing-temperature cultivator
- High-speed centrifuge
- Absorbent paper
- Single or multi-channel pipettes with high precision and disposable tips.
- Precision pipettes to deliver 2 μL to 1 mL volumes.
- Clean tubes
- Deionized or distilled water
- Microplate reader with 450 ± 10 nm wavelength filter

Precautions

- After opening the package, please check that all components are complete.
- 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.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells.
- If crystals have formed in the Wash Solution concentrate (25×), warm to room temperature and mix gently until the crystals are completely dissolved.
- Prepare standards within 15 minutes before assay. This standard can only be used once.
- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent color development. Warning TMB is toxic avoid direct contact with hands. Dispose of properly. If a dark blue color develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells. Dispense the TMB solution within 15 minutes following the washing of the microtiter plate.
- It is highly recommended to use the remaining reagents within 1 month provided this is prior to the expiration date of the kit. For the expiration date of the kit, please refer to the label on the kit box.

Specimen Collection

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.

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

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

Cell culture supernatant and other biological fluids Centrifuge samples for 20 minutes at 1000 × g. 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.

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 detached with trypsin, and collected by centrifugation at 1000 × g for 5 minutes (suspension cells can be collected by centrifugation directly).
2. Wash cells three times in pre-cooled PBS.
3. Cells were then resuspended in fresh lysis buffer with concentration of 10^7 cells/mL. If it is necessary, the cells could be subjected to ultrasonication till the solution is clarified.
4. Centrifuge at 1500×g for 10 minutes at 2-8°C to remove cellular debris. Assay immediately or aliquot and store at ≤-20°C.

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

1. Tissues were rinsed in pre-cooled PBS to remove excess blood thoroughly and weighed before homogenization.
2. Minced 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. The resulting suspension was sonicated with an ultrasonic cell disrupter till the solution was clarified.
4. Then, the homogenates were centrifuged for 5 minutes at 10000 x g and collect the supernatant and assay immediately or aliquot and store at ≤-20 °C.
   Note: Tissue homogenate samples 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.

Cerebrospinal fluid (CSF) - Remove particulates by centrifugation and assay immediately or aliquot and store samples 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.

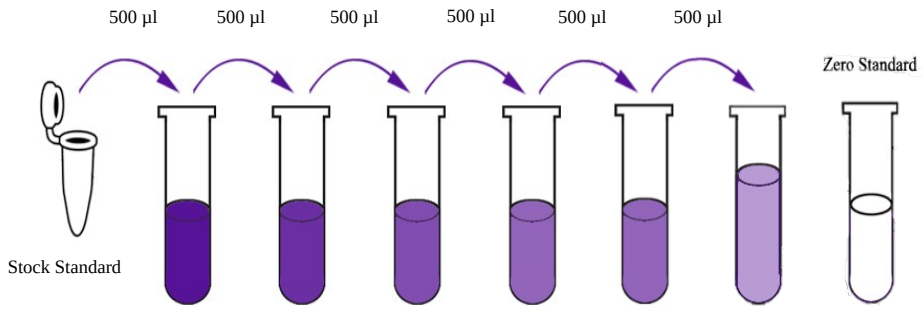
Note

- Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination. Avoid repeated freeze/thaw cycles.
- Sample hemolysis will influence the result, so hemolytic specimen should not be used.
- When performing the assay, bring samples to room temperature.
- If the concentration of the test material in your sample is higher than that of the standard product, please make the appropriate multiple dilution according to the actual situation (it is recommended to do preliminary experiment to determine the dilution ratio.

Reagent Preparation

- Bring all kit components and samples to room temperature (18-25°C) before use.
- If the kit will not be used up in one time, please only take out strips and reagents for present experiment, and save the remaining strips and reagents as specified.
- Dilute the 25x wash buffer into 1x working concentration with double distilled water.
- **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 200 pg/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 **200 pg/mL, 100 pg/mL, 50 pg/mL, 25 pg/mL, 12.5 pg/mL, 6.25 pg/mL, 3.13 pg/mL,** and the last EP tubes with Standard Diluent is the Blank as 0 pg/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.



- **Biotinylated Antibody and 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.
- **TMB substrate** - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Notes

1. 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).
2. 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 to allow the liquid on the tube wall or bottle cap to settle to the bottom of the tube. Before use, 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.
3. 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.
4. 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.
5. 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.
6. TMB is light-sensitive, avoid prolonged exposure to light. Dispense the TMB Solution within 15 minutes following the washing of the microtiter plate. In addition, avoid contact between

TMB 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.

7. Bacterial or fungal contamination of either samples or reagents or cross-contamination, between reagents may cause erroneous results.

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 sealer. 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. Remove 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.

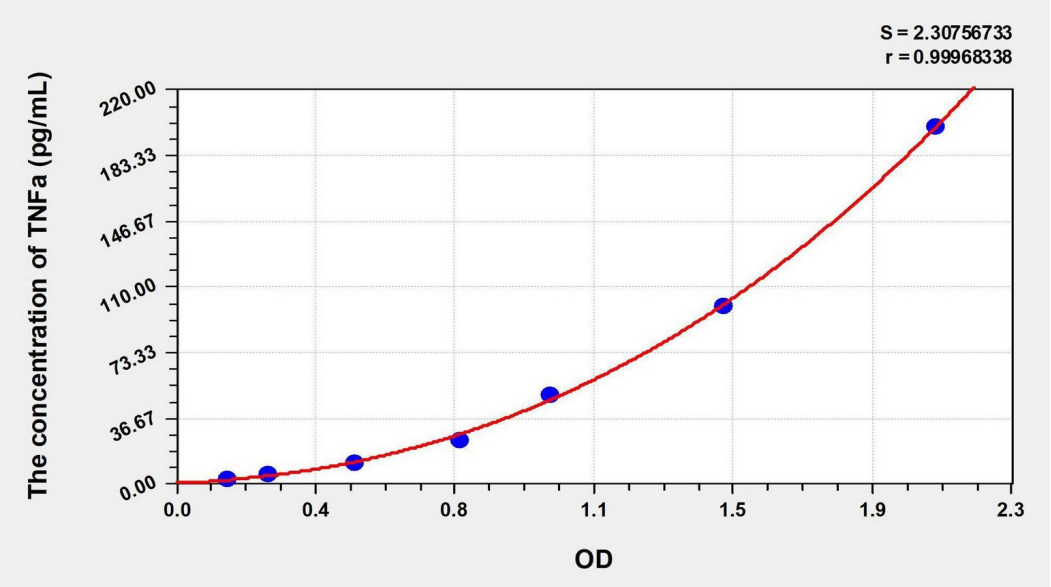
   (b) Pay attention to pouring the washing liquid directly to ensure that the washing liquid does not contaminate other wells.
3. Add 100 µL of Biotinylated Antibody Working Solution to each well, cover the wells with the plate sealer and incubate for 50 minutes at 37°C.
4. Repeat the aspiration, wash 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 sealer. Incubate for 20 minutes at 37°C (Don't exceed 30 minutes). Protect from light. 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. Remove 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 Result

Average the duplicate readings for each Standard, Control, and Samples and subtract the average zero Standard optical density. Construct a Standard curve with the Zebrafish TNFa 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.

Typical Data

This standard curve is only for demonstration purposes. A standard curve should be generated with each assay.



Note: This graph is for reference only

Concentration (pg/mL)	OD	Corrected OD
200	2.146	2.08
100	1.563	1.497
50	1.088	1.022
25	0.841	0.775
12.5	0.553	0.487
6.25	0.315	0.249
3.13	0.206	0.14
0	0.066	0.000

Summary

1. Prepare all reagents, samples and standards.
2. Add 100 µL Standard or sample per well. Incubate for 80 min at 37°C. Wash the plate 3 times.
3. Add 100 µL Biotinylated Antibody Working Solution. Incubate for 50 min at 37°C. Wash the plate 3 times.
4. Add 100 µL Streptavidin-HRP Working Solution. Incubate for 50 min at 37°C. Wash the plate 5 times.
5. Add 90 µL TMB Substrate Solution. Incubate for 20 min at 37°C, protected from light.
6. Add 50 µL Stop Reagent and the color develops.
7. Read the OD value at 450 nm immediately.

Shipping Direction - Temperature

Maintain temperature between 2°C to 8°C during transportation in unopened packaging.

Troubleshooting

Possible Case	Solution
High Background	
• Improper washing • Substrate was contaminated • Non-specific binding of antibody • Plate are not sealing incompletely • Incorrect incubation temperature • Substrate exposed to light prior to use • Contaminated wash buffer	• Increasing duration of soaking steps • Replace. Substrate should be clean and avoid crossed contamination by using the sealer • Replace another purified antibody or blocking buffer • Make sure to follow the instruction strictly • Incubate at room temperature • Keep substrate in a dark place • Use a clean buffers and sterile filter
Weak Signal	
• Improper washing • Incorrect incubation temperature • Antibodies are not enough • Reagent are contaminated • Pipettes are not clean	• Increasing duration of soaking steps • Incubate at room temperature • Increase the concentration of the antibody • Use new one • Pipette should be clean
No Signal	
• Reagent are contaminated • Sample prepared incorrectly • Antibodies are not enough • Wash buffer contains sodium azide • HRP was not added	• Use new one • Make sure the sample workable/dilution • Increase the antibody concentration • Use a new wash buffer and avoid sodium azide in it • Add HRP according to the instruction
Poor Precision	
• Imprecise/ inaccurate pipetting • Incomplete washing of the wells	• Check/ calibrate pipettes • Make sure wells are washed adequately by filling the wells with wash buffer and all residual antibody solutions crossed well before washing.

This product is for research use only, not for use in diagnosis procedures.
It's highly recommended to read this instruction entirely before use.

For more information:
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