(For Research Use Only. Not For Use In Diagnostic Procedures!)

FineTest®

Human H3cit-NE (neutrophil extracellular trap) ELISA kit

Catalogue No.: EH5035

Revision: V4.0

Size: 48T/96T

Please read the manual carefully before use. Please feel free to contact us, if you have any questions.

Tel	027-87384275
Email	fine@fn-test.com
Website	https://www.fn-test.com/

Please provide the batch number (see kit label) for more rapid response and services.

It's strongly recommended to use this kit within the expiry date printed on the kit label.

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Technical support related documents

Title of Document	Sample preparation guide	Experimental operation procedure	TMB color rendering control
Website	https://www.fn-test.com/content/uploads/2022/06/ELISA-Sample-Preparation-Protocol-2022.6.6.pdf	https://www.fn-test.com/videos/elisa-test/	https://www.fn-test.com/videos/targeted-control-of-tmb-coloring/
QuickMark			

Product Features

Application	For qualitative detection of the presence NETS		
Reactivity	Human	Detection Method	Sandwich
Detection Duration	4 hours(excluding balancing and sample preparation)		
Samples needed for single well(Max)	100µL of culture supernatant, 100µL of culture lysate, and 50ul serum and plasma,50ul other liquid samples that may contain NETS		
Specificity	NETS		
Storage	2-8°C (for sealed box), please do not freeze! See kit label for expiry date		

Background

NETs are a network of DNA fibers, histones and antibacterial proteins secreted by activated neutrophils that are responsible for trapping and killing extracellular pathogens and play a protective role in antibacterial defense.

Principle of the Assay

- This kit was based on sandwich enzyme-linked immune-sorbent assay technology. Capture antibody (anti Citrullinated histone H3) was pre-coated onto the 96-well plate. The biotin conjugated antibody (anti Neutrophil elastase) was used as the detection antibody. The Positive control and samples were added to the wells subsequently. After incubation, unbound conjugates were removed by wash buffer. Then, biotinylated detection antibody was added to bind with antigen conjugated on coated antibody. After washing off unbound conjugates, HRP-Streptavidin was added. After a third washing, TMB substrates were added to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that turned yellow after adding acidic stop solution. Read the O.D. absorbance at 450nm in a microplate reader. The relative concentration of the sample can be compared by OD value.

- **Kit Components and Storage**

The sealed kit can be stored at 2-8 ℃. The storage condition for opened kit is specified in the table below:

No.	Item	Size(48T)	Size(96T)	Storage Condition for Opened Kit
E001	ELISA Microplate(Dismountable)	8×6	8×12	Put the rest strips into a sealed foil bag with the desiccant. Stored for 1 month at 2-8°C; Stored for 6 month at -20°C
E002	Lyophilized Positive control	1vial	2vial	Put the rest positive control into a desiccant bag. Stored for 1 month at 2-8°C; Stored for 6 month at -20°C
E003	Biotin-labeled Antibody(Concentrated, 100X)	60ul	120ul	2-8°C (Avoid Direct Light)
E034	HRP-Streptavidin Conjugate(SABC, 100X)	60ul	120ul	
E024	TMB Substrate	5ml	10ml	
E039	Sample Dilution Buffer	10ml	20ml	2-8°C
E040	Antibody Dilution Buffer	5ml	10ml	
E049	SABC Dilution Buffer	5ml	10ml	
E026	Stop Solution	5ml	10ml	
E038	Wash Buffer(25X)	15ml	30ml	
E006	Plate Sealer	3 pieces	5 pieces	
E007	Product Description	1 copy	1 copy	

Note: The reagent bottle contains slightly more reagent than indicated on the label. Please use pipette accurately measure and do proportional dilution.

Required Instruments and Reagents

1. Microplate reader (wavelength: 450nm)
2. 37°C incubator (CO_2 incubator for cell culture is not recommenced.)
3. Automated plate washer or multi-channel pipette/5ml pipettor (for manual washing purpose)
4. Precision single (0.5-10μL, 5-50μL, 20-200μL, 200-1000μL) 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

Sample Collection and Storage

The following sample processing steps are concise operations. For detailed sample preparation guideline, please refer to the QuickMark or the link (https://www.fn-test.com/content/uploads/2022/06/ELISA-Sample-Preparation-Protocol-2022.6.6.pdf).

1. Cell Culture Supernatant

Collect the supernatant: Centrifuge at 2500 rpm at 2-8℃ 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.

2.Cell Lysate

2.1 Suspension Cell Lysate: Centrifuge at 2500 rpm at 2-8℃ 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.

2.2. 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).

2.3. At the end of lysate or ultrasonic disruption, centrifuge at 10000rpm at 2-8℃ 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.

3. Other Biological Sample

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

Recommended reagents for sample preparation: Cat No: E051 100mM PMSF protease inhibitor, Cat No: E050 FineTest Lysis Buffer (for ELISA).

Notes for Samples

1. The best sample storage condition: less than 5 days at 2-8℃; within 6 months at -20℃; within 2 years at -80℃. Stored in liquid nitrogen for a longer storage. When melting frozen samples, rapid water bath at 15-25℃ can decrease the effect of ice crystal (0℃) on the sample. After melting, centrifuge to remove the precipitate, and then mix well.

2. 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.

Precautions for Kits

1. When using different Elisa kits, labeling is required to avoid mixed components and failed assay.

2. After opening the kit, please refer to the table of storage condition for coated plate and Positive control (Dampness may decrease the activity.). Other reagents can be stored at 2-8℃. If any component is missing or damaged during the assay or storage, please contact us for ordering a new one to replace.(e.g. E002 lyophilized Positive control)

3. Sterile and disposable tips are required during the assay. After use, the reagents bottle cap has to be tightened to avoid the microbial contamination and evaporation.

4. While manual washing, please keep tips or pipettors for adding wash buffer away from the well. Insufficient washing or contamination easily causes false positive and high background.

5. During the assay, prepare required reagents for next step in advance. After washing, add the reagent into the well in time to avoid dryness. Otherwise, dry plate will result in the failed assay.

6. Before confirmation, reagents from other batches or sources should not be used in this kit.

7. Don't reuse tips and tubes to avoid cross contamination.

8. After loading, seal the plate to avoid the evaporation of the sample during incubation. Complete the incubation process at recommended temperature.

9. 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.

Recommended Sample Dilution Ratio

Please refer to the following table of recommended dilution ratio for NETS samples for reference. (ND: Not Detected)

Sample Type	Condition	OD450	control OD450
Human peripheral blood leukocyte cells (3 x 10^6 cells/mL) Cell Culture Supernatant(48h) 100ul	Unstimulated	0.320	positive（2.326）
	Stimulated LPS(1ug/ml)	1.751	negative（0.127）

If other dilution ratio for your sample model is required, please refer to the universal dilution ratio below. (The ratio is suitable for single-well assay. For duplicate assay, please follow the calculation: volume of sample and diluent x number of duplicate well)

For 2 fold dilution (1/2): One step dilution. Add 60µL sample into 60µL sample diluent and mix gently.

For 5 fold dilution (1/5): One step dilution. Add 24µL sample into 96µL sample diluent and mix gently.

For 10 fold dilution (1/10): One step dilution. Add 12µL sample into 108µL sample diluent and mix gently.

For 20 fold dilution (1/20): One step dilution. Add 6µL sample into 114µL sample diluent and mix gently.

For 50 fold dilution (1/50): One step dilution. Add 3µL sample and 47µL normal saline (0.9% NaCl) into 100 µL sample diluent and mix gently.

For 100 fold dilution (1/100): One step dilution. Add 3µL sample and 177µL normal saline into 120µL sample diluent and mix gently.

For 1000 fold dilution (1/1000): Two step dilution. Create a 50-fold dilution first (normal saline is used throughout the dilution). Then, create a 20-fold dilution and mix gently.

For 10000 fold dilution (1/10000): Two step dilution. Create a 100-fold dilution first (normal saline is used throughout the dilution). Then, create the same dilution again and mix gently.

For 100000 fold dilution (1/100000): Three step dilution. Create a 50-fold dilution and 20-fold dilution respectively (normal saline is used in the first two steps.) Finally, create a 100-fold dilution and mix gently.

Notes: The volume in each dilution is not less than 3µL. Dilution factor should be within 100 fold. Mixing during dilution is required to avoid foaming.

Reagent Preparation and Storage

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

1. Wash Buffer

Dilute 30ml (15ml for 48T) concentrated wash buffer to 750ml (375ml for 48T) wash buffer with deionized or distilled water and mix well. (The recommended resistivity of ultrapure water is 18MΩ.) 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℃.

Crystals formed in the concentrated wash buffer can be heated by water bath at 40℃ till complete dissolution. (Heating temperature should be below 50℃.) 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℃ within 48h.

2. Lyophilized Positive control and negative control

2.1. Centrifuge positive control tube for 1min at 10000xg. Label it as positive control tube.

2.2. Add 1ml sample dilution buffer into the positive control tube. Tighten the tube cap and Let it stand for 2min 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 1000xg, making the liquid towards the bottom of tube and removing possible bubbles.

2.4. **negative control:** Sample diluent, negative culture medium, or negative human blood as negative quality control products, priority to use sample diluent as negative quality control.

Notes: Store the positive control tube with dissolved control at 2-8℃ and use it within 12h.

3. Preparation of Biotin-labeled Antibody Working Solution

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

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

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

3.3. Dilute the biotinylated detection antibody with antibody dilution buffer at 1:99 and mix them thoroughly. (e.g. Add 10ul concentrated biotin-labeled antibody into 990ul antibody dilution buffer.)

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

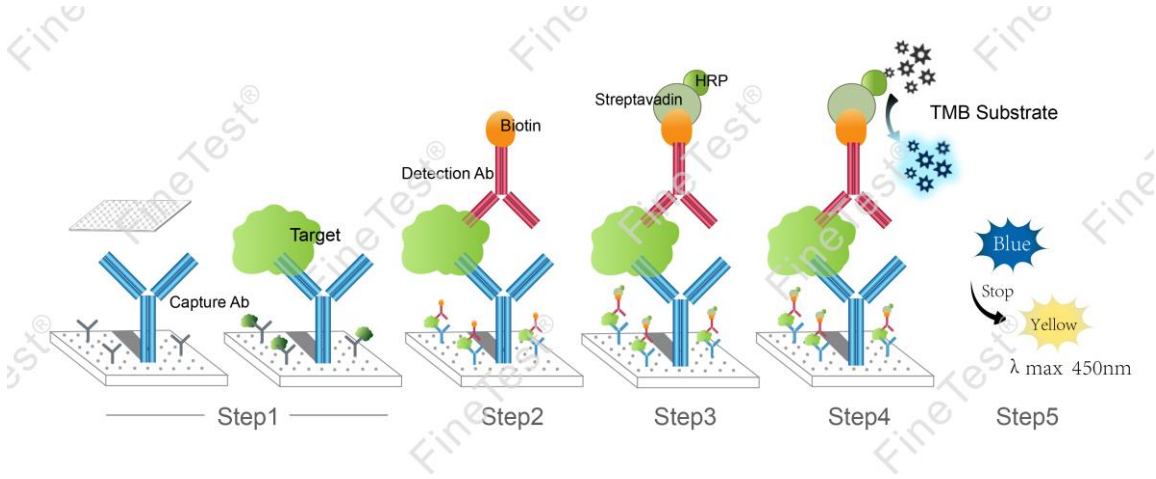
The working solution should be prepared within 30min before the assay and can't be stored for a long time.

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

4.2. Centrifuge for 1min at 1000xg 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 10ul concentrated SABC into 990ul SABC dilution buffer.)

Assay Procedure Summary



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

Washing: Wash the plate twice without immersion.

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

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

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

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

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.

Detailed Assay Procedure

When diluting samples and reagents, they must be mixed completely.

1. Set positive control, pilot samples, negative control (blank) wells on the pre-coated plate respectively, and then, records their positions. It's recommended to measure each control and sample in duplicate to decrease experimental errors.

2. Control and samples loading: Aliquot 100ul of into positive control well. Also add 100ul sample dilution buffer into the negative control (blank) well. Then, add 100ul pilot samples into each sample well. Seal the plate and 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 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 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 incubate at 37°C in dark within 10-20 minutes. Run the microplate reader and preheat for 15min. (Notes: 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 control wells. Weaker or stronger color intensity is unacceptable. Please refer to TMB color rendering control in page 2 or QR code for detail.)

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 and calculate.

Results

1. Result of contrast by the mean OD450 value of the duplicate readings for each control and sample. Then, obtain the corrected OD450 by subtracting the OD450 blank. The sample value(subtracting the OD450 blank) ≥ 0.1 is positive, and the higher the concentration of Nets, the higher the value.

Typical Data

The following assay data are provided for reference, since experimental environment and operation are different. The establishment of control depends on your own assay.

control	color development time	OD-1	OD-2	Average
negative control	5 mins	0.076	0.075	0.076
	10 mins	0.102	0.105	0.104
	20 mins	0.132	0.139	0.135
positive control	5 mins	0.737	0.767	0.752
	10 mins	1.229	1.279	1.254
	20 mins	2.417	2.515	2.466

Precision

Intra-assay Precision: samples with low, medium and high concentration are tested 20 times on same plate.

Inter-assay Precision: samples with low, medium and high concentration are tested 20 times on three different plates.

Item	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean(OD450)	0.60	1.073	2.226	0.61	1.122	2.358
CV(%)	6.7	5.6	4.7	6.2	5.3	4.6

Stability

Perform the stability test for the sealed kit at 37°C and 2-8°C and get relevant data.

Elisa kit(n=5)	37°C for 1 month	2-8°C for 6 months
Average (%)	80	95-100

ELISA Troubleshooting

If the ELISA result is unsatisfied, please take a screenshot for the staining result and store the OD data. Keep used strips as well the rest reagents. Contact us to solve your problem with the kit's catalogue number and batch number. You can also refer to the following table to check the reason.

Problem	Possible Causes	Solutions
control without signal	Incorrect order for adding reagents	Confirm the required reagent added in each step. Also repeat the assay and verify.
	Use components 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.
Samples without signal	The amount of pilot sample is lower than the detection sensitivity.	Decrease dilution ratio or concentrate the sample.
	The detection target is incompatible with the buffer.	Verify the compatibility of sample storage buffer with the pilot sample.
	Incorrect preparation of sample	Please refer to sample preparation guideline and regularly store.
	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.
control with low signal	control are improperly reconstituted.	Before opening, shortly centrifuge the lyophilized control tube till complete dissolution.
	control have been degraded.	Follow suggested storage conditions for control.
	When pipetting, the required volume	Use calibrated pipette and correct pipetting

	is incorrect or inaccurate.	method.
	Expired kit	Don't use expired products.
	Improper storage	Follow suggested storage conditions for all components.
	The well is over dried.	The assay and sample loading process can't be terminated. Especially after washing the plate, add reagents immediately. Seal the plate during incubation.
	Slow colorimetric reaction	Before use, equilibrate the whole bottle of TMB substrate for 30min at 37°C. Extend the incubation time.
	The wavelength of the microplate reader is incorrect.	Check the wavelength and read the OD450 value again.
	The well is washed excessively.	Follow suggested washing times in this manual.
High Background	Insufficient washing	Follow suggested washing times in this manual.
	Wash buffer is contaminated.	Use the prepared wash buffer immediately. During manual washing, add wash buffer without touching the well.
	Too many detection reagents or higher concentration.	Use calibrated pipette and correct pipetting method.
	Reading of assay result is not in time.	Read the assay result immediately after adding the stop solution.
	TMB substrate is incubated in strong light.	During colorimetry, incubate in the dark.

Declaration

1. Limited to current conditions and scientific techniques, all raw materials are not completely identified and analyzed. This product may have a technology-related quality risk.

2. During the Elisa kit development, some endogenous interferons(not all) in the biological sample have been removed or decreased.

3. The final assay result is related to the validity of reagents, experimental operation and environment. FineTest is only responsible for this kit, excluding sample consumption during using this kit. Before use, please consider and prepare enough samples required by the assay.

4. To get a satisfied assay result, please use all reagents offered by this kit. Don't use any product from other vendors. Strictly follow instructions of this manual.

5. During assay procedure, incorrect reagents preparation and parameter setting of the microplate reader may result in the abnormal result. Before assay, please read this manual carefully and adjust instruments.

6. Even if the assay is performed by the same person, results in two independent assays may be different. Thus, each step in the assay should be controlled to ensure the reproducibility.

7. Before delivery, this kit is subject to the strict QC. Influenced by transportation conditions and experimental devices, the assay result got by the customer may be different from original data. Inter-assay CV between different batches may be caused by reasons before.

8. This kit is not compared to similar kits from other vendors or methods for testing the same detection target. Thus, assay results may be inconsistent.

9. This kit allows for research use only. For IVD or other purposes, FineTest is not responsible for relevant consequences and doesn't bear any legal liability.