（FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSIS !）

Catalog No：E-BC-K884-M

Specification：48T (32 samples)/96T(80 samples)

Measuring instrument：Microplate reader (360 - 400 nm)

Detection range：0.31-18.33 U/L

Elabscience® NETosis Colorimetric Assay Kit

This manual must be read attentively and completely before using this product.
If you have any problem, please contact our Technical Service Center for help：

Toll-free: 1-888-852-8623
Tell: 1-832-243-6086
Fax: 1-832-243-6017
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

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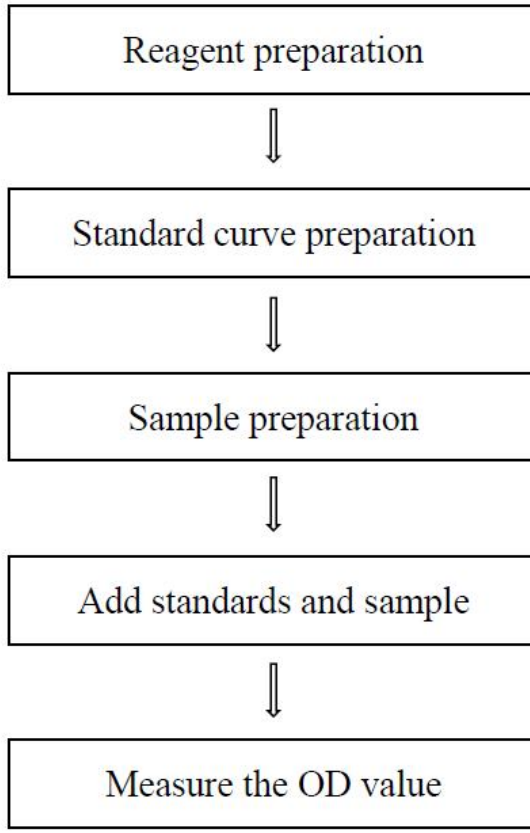
Assay summary



Intended use

This kit can be used to measure the process of NETosis in cell samples.

Detection principle

NETosis is an inflammatory cell death mode of neutrophils. Activated neutrophils capture and kill pathogens by releasing Neutrophil extracellular traps (NETs), which are composed of deaggregated chromatin and intracellular granule proteins. The formation of NETs is accompanied by the death of neutrophils. This new type of death is different from apoptosis and necrosis and is called NETosis. There are many inducers of NETosis, such as pathogens (bacteria, fungi, viruses, etc.), activated platelets, chemokines (interleukins, granulocyte colony-stimulating factor, transforming growth factor β, etc.), artificial ingredients and calcium ionophore, etc. These inducers can promote the key proteins of NETosis, Neutrophil Elastase (NE), Myeloperoxidase (MPO) and Peptidearginine Deaminase IV (PAD4) transcription and translation.

This method evaluated the degree of NETosis by detecting the enzyme activity of NE.

Kit components & storage

Item	Component	Size 1(48 T)	Size 2(96 T)	Storage
Reagent 1	Protein Reagent	1.2 g × 1 vial	2.4 g × 1 vial	-20℃, 12 months
Reagent 2	Saline Solutiont	0.12 mL × 1 vial	0.24 mL × 1 vial	-20℃, 12 months
Reagent 3	PMA Stock Solution	0.01 mL × 1 vial	0.02 mL × 1 vial	-20℃, 12 months shading light
Reagent 4	A23187 Stock Solution	0.02 mL × 1 vial	0.04 mL × 1 vial	-20℃, 12 months shading light
Reagent 5	Scavenger	0.02 mL × 1 vial	0.04 mL × 1 vial	-20℃, 12 months shading light
Reagent 6	Stop Solution	0.55 mL × 1 vial	1.1 mL × 1 vial	-20℃, 12 months
Reagent 7	Substrate	0.55 mL × 1 vial	1.1 mL × 1 vial	-20℃, 12 months shading light
Reagent 8	Buffer Solution	5.5 mL × 1 vial	11 mL × 1 vial	-20℃, 12 months
Reagent 9	55 mmol/L Standard Solution	0.06 mL × 1 vial	0.06 mL × 1 vial	-20℃, 12 months shading light
	Microplate	48 wells	96 wells	No requirement
	Plate Sealer	2 pieces		

Note: The reagents must be stored strictly according to the preservation conditions in the above table. The reagents in different kits cannot be mixed with each other. For a small volume of reagents, please centrifuge before use, so as not to obtain sufficient amount of reagents.

Materials prepared by users

Instruments:

Microplate reader (360 - 400 nm, optimum wavelength: 380 nm), Vortex mixer, Incubator

Reagents:

Basal medium, DMSO

Consumptive material:

0.22 μm filter membrane, Syringe

Reagent preparation

① Equilibrate all the reagents to 25°C before use.

② The preparation of assay buffer solution：

Mix well 100 μL of culture medium with 1 g of protein reagent and 0.1 mL of saline solution, the solution was stirred, filtered through a 0.22 μm filter membrane, and then packaged and placed in a sterile environment at 2-8°C for use. Store at 2-8°C for 2 weeks.

③ The preparation of PMA working solution：

Before testing, please prepare sufficient PMA working solution. For example, prepare 5 mL of PMA working solution (mix well 0.001 mL of PMA stock solution and 4.999 mL of analysis buffer solution).Store at -20°C for 3 days protected from light.

④ The preparation of A23187 working solution：

Before testing, please prepare sufficient A23187 working solution. For example, prepare 1 mL of A23187 working solution (mix well 0.01 mL of A23187 stock solution and 0.99 mL of analysis buffer solution).Store at -20°C for 3 days protected from light.

⑤ The preparation of scavenger working solution：

Before testing, please prepare sufficient scavenger working solution. For example, prepare 0.5 mL of scavenger working solution (mix well 0.003 mL of scavenger and 0.497 mL of analysis buffer solution).The enzyme working solution should be prepared on spot.

⑥ The preparation of substrate working solution：

Before testing, please prepare sufficient substrate stock solution. For example, prepare 20 μL of substrate working solution (mix well 5 μL of substrate and 15 μL of DMSO) as substrate stock solution. Please prepare sufficient substrate working solution. For example, prepare 25 μL of substrate working solution (mix well 10 μL of substrate stock solution and 15 μL of buffer solution).Store at 2-8°C for 3 days protected from light.

⑦ The preparation of 550 μmol/L standard solution：

Before testing, please prepare sufficient 550 μmol/L standard solution. For example, prepare 1000 μL of scavenger working solution (mix well 10 μL of 55 mmol/L standard solution and 990 μL of double distilled water).Store at 2-8°C for 1 week protected from light.

⑧ The preparation of standard curve：

Always prepare a fresh set of standards. Discard working standard dilutions after use.

Dilute 550 μmol/L standard with double distilled water diluent to a serial concentration. The recommended dilution gradient is as follows: 0, 110, 220, 275, 330, 385, 440, 550 μmol/L. Reference is as follows:

Item	①	②	③	④	⑤	⑥	⑦	⑧
Concentration (μmol/L)	**0**	**110**	**220**	**275**	**330**	**385**	**440**	**550**
550 μmol/Lstandard (μL)	0	40	80	100	120	140	160	200
Double distilled water (μL)	200	160	120	100	80	60	40	0

Operating steps

Sample preparation:

① Neutrophils (cell density should be at least 1×10^6 cells /mL) were resuspended with assay buffer solution, take 0.9 mL of cell suspension into EP tubes, add

0.1 mL of PMA working solution or A23187 working solution, and incubated for 4-6 h at 37°C.

② Discarded the supernatant, and the cells were washed twice with assay buffer solution prewarmed at 37°C (add 1 mL each time, centrifuged at 300 × g for 5 min at 4°C, and the supernatant was discarded).

③ Add 0.5 mL of scavenger working solution, incubated at 37°C for 30 min, take the supernatant into new EP tube, add 0.01 mL of stop solution, and centrifuged at 300×g for 5 min at 4°C. Take the supernatant into EP tube, keep it on ice for detection (Store at 4°C for a week or -20°C for 3 months). Precipitate and homogenize the cells for detection (homogenize 1×10^6 cells in 200 μL normal saline (0.9%NaCl) with a ultrasonic cell disruptor at 4℃. Centrifuge at 10000×g for 10 min at 4℃ to remove insoluble material. Collect supernatant and keep it on ice for detection).

Detection steps:

① Standard well: Add 20 μL of standard with different concentrations into the corresponding wells.

Sample well: Add 20 μL of sample into the corresponding wells.

② Add 100 μL of substrate working solution into the corresponding wells.

③ Mix fully with microplate reader for 5 s, incubate at 37°C for 30 min, measure the OD value of each well at 380 nm with microplate reader.

Calculation

The standard curve:

1. Average the duplicate reading for each standard.

2. Subtract the mean OD value of the blank (Standard #①) from all standard readings. This is the absoluted OD value.

3. Plot the standard curve by using absoluted OD value of standard and correspondent concentration as y-axis and x-axis respectively. Create the standard curve (**y = ax + b**) with graph software (or EXCEL).

The sample:

1. Cell samples:

Definition: The amount of enzyme in 1 g of cell protein in reaction system that produce 1 μmol production at 37 ℃ for 1 min is defined as 1 unit.

$$\frac{\text{NE activity}}{\text{(U/gprot)}} = (\Delta A - b) \div a \div C_{pr} \div T$$

[Note]

ΔA: Absolute OD, $OD_{Sample} - OD_{Blank}$.

C_{pr}: Concentration of protein in sample, gprot/L.

T: Reaction time, 30 min.

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Appendix I Performance Characteristics

1. Parameter:

Intra-assay Precision

Three 293T cell samples were assayed in replicates of 20 to determine precision within an assay (CV = Coefficient of Variation).

Parameters	Sample 1	Sample 2	Sample 3
Mean (U/L)	5.40	2.60	3.10
%CV	3.2	3.8	2.1

Inter-assay Precision

Three 293T cell samples were assayed 20 times in duplicate by three operators to determine precision between assays.

Parameters	Sample 1	Sample 2	Sample 3
Mean (U/L)	5.40	2.60	3.10
%CV	6.8	7.4	4.8

Recovery

Take three samples of high concentration, middle concentration and low concentration to test the samples of each concentration for 6 times parallelly to get the average recovery rate of 97.7%.

	Standard 1	Standard 2	Standard 3
Expected Conc. (U/L)	5.4	2.6	3.1
Observed Conc. (U/L)	5.1	2.5	3.1
Recovery rate (%)	95	98	100

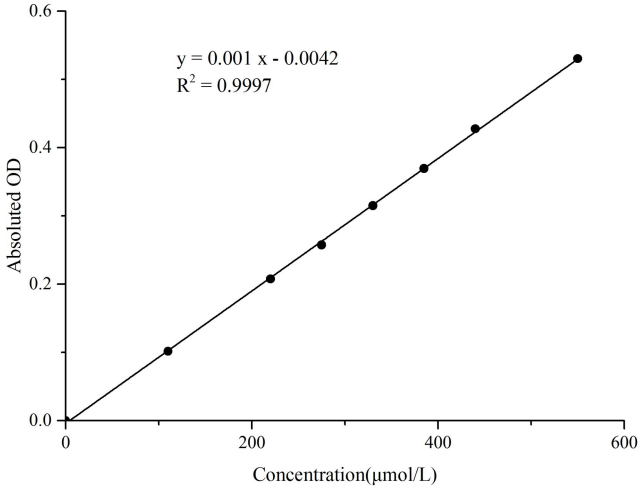
Sensitivity

The analytical sensitivity of the assay is 0.31 U/L. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 20 times, and calculating the corresponding concentration.

2. Standard curve:

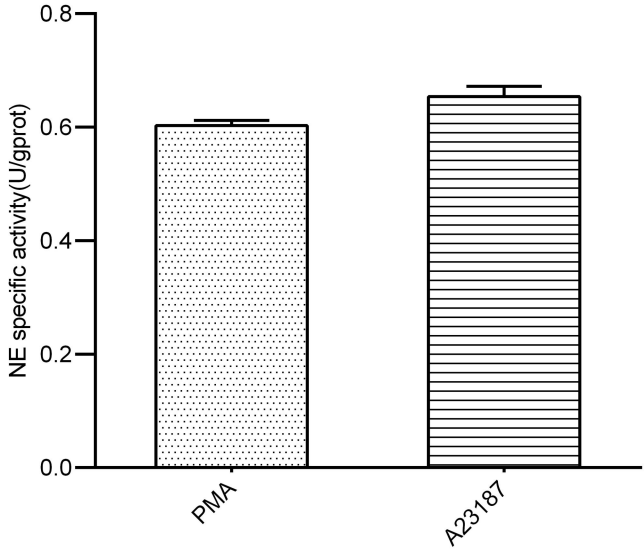
As the OD value of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique or temperature effects), so the standard curve and data are provided as below for reference only:

Concentration (μmol/L)	0	110	220	275	330	385	440	550
OD value	0.157	0.256	0.355	0.409	0.467	0.526	0.581	0.686
	0.157	0.261	0.374	0.420	0.477	0.527	0.588	0.689
Average OD	0.157	0.259	0.365	0.415	0.472	0.527	0.585	0.688
Absoluted OD	0	0.102	0.208	0.258	0.315	0.370	0.428	0.531



Appendix II Example Analysis

The NE activity in neutrophils PMA and A23187 induced by PMA and A23187



Statement

1. This assay kit is for Research Use Only. We will not response for any arising problems or legal responsibilities causing by using the kit for clinical diagnosis or other purpose.
2. Please read the instructions carefully and adjust the instruments before the experiments. Please follow the instructions strictly during the experiments.
3. Protection methods must be taken by wearing lab coat and latex gloves.
4. If the concentration of substance is not within the detection range exactly, an extra dilution or concentration should be taken for the sample.
5. It is recommended to take a pre-test if your sample is not listed in the instruction book.
6. The experimental results are closely related to the situation of reagents, operations, environment and so on. Elabscience will guarantee the quality of the kits only, and NOT be responsible for the sample consumption caused by using the assay kits. It is better to calculate the possible usage of sample and reserve sufficient samples before use.