

Version 3 Last updated 16 March 2022

# ab235979 NETosis Assay Kit

For the study of the process of NETosis *ex vivo*.

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

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## 1. Overview

NETosis Assay Kit (ab235979) provides a simple and fast method for studying the process of NETosis *ex vivo*.

In this kit, primary neutrophils are stimulated to release NETs with either PMA or a calcium ionophore (both included). Unbound neutrophil elastase is washed away following NET generation. Following digest of NET DNA by S7 nuclease, the supernatant containing neutrophil elastase is added to a substrate, which is selectively cleaved by elastase to yield a 4-nitroaniline product that adsorbs light at 405 nm. Enough reagents are provided to test 24 sample conditions for NET production, with analysis in duplicate.

**Δ Note:** This kit does not depend upon the DNA component of neutrophil extracellular traps (NETs), as DNA release can occur independently of NETosis.

Prepare reagents, substrate, standard curve and samples.



Add standards, samples and substrate to the microplate.



Cover plate with cover sheet and incubate for 1-2 hours at 37°C.



Remove cover sheet and read absorbance at 405 nm.

## 2. Materials Supplied and Storage

Store kit at -20°C immediately on receipt and check below for storage for individual components. Kit can be stored for 1 year from receipt, if components have not been reconstituted.

Aliquot components in working volumes before storing at the recommended temperature.

Avoid repeated freeze-thaws of reagents.

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
96-Well Plate (Colorimetric Assay)	1 unit	RT	4°C
96-Well Plate Cover	1 unit	RT	4°C
A-23187 (25 mM) Assay Reagent	50 µL	-20°C	-20°C
Bovine Serum Albumin Assay Reagent	5 g	4°C	4°C
Calcium Chloride (1 M) Assay Reagent	1 mL	RT	4°C
Cell-Based PMA (1 mM)	50 µL	-20°C	-20°C
EDTA (500 mM) Assay Reagent	1 mL	RT	4°C
Human Neutrophil Elastase Assay Reagent	50 µL	-20°C	-20°C
NET Assay Neutrophil Elastase Substrate	2 vials (250 µL)	-20°C	-20°C
S7 Nuclease Assay Reagent	50 µL	-20°C	-20°C

### 3. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at O.D. 400 nm to 420 nm
- RPMI cell culture medium
- A source of NET-producing cells (e.g., human peripheral blood neutrophils)
- 24 well plate with clear flat bottom
- PBS
- NET Assay Buffer:

To prepare NET Assay Buffer, combine 500 mL of RPMI 1640 base medium (not provided) with 5 g Bovine Serum Albumin and 500  $\mu$ L of 1 M Calcium Chloride. NET Assay Buffer is not intended to be sterile and does not need to be prepared or used in a tissue culture hood. For storage of unused NET Assay Buffer, sterile filter, aliquot and store at -20°C.

**Δ Note:** Serum contains DNAase that will digest NETs and should be avoided if possible.

**Δ Note:** Pre-warm to 37°C prior to cell stimulation and addition of nuclease to ensure rapid activation and subsequent nuclease activity.

## 4. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:

[www.abcam.com/assaykitguidelines](http://www.abcam.com/assaykitguidelines)

For typical data produced using the assay, please see the assay kit datasheet on our website.

## 5. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

### 5.1 96-Well Plate (Colorimetric Assay)

1. Ready to use as supplied.

### 5.2 96-Well Plate Cover

1. Ready to use as supplied.

### 5.3 A-23187 (25 mM) Assay Reagent

1. Prior to use add 10  $\mu$ L of 25 mM A-23187 to 1 mL of pre-warmed NET Assay Buffer to make a 10X working stock.

### 5.4 Bovine Serum Albumin Assay Reagent

1. Ready to use as supplied.

### 5.5 Calcium Chloride (1 M) Assay Reagent

1. Ready to use as supplied.

### 5.6 Cell-Based PMA (1 mM)

1. Prior to use, add 1  $\mu$ L of 1 mM PMA to 5 mL of pre-warmed NET Assay Buffer to make a 10X working stock.

**ΔNote:** PMA is a potential carcinogen. Wear gloves when using this reagent.

### 5.7 EDTA (500 mM) Assay Reagent

1. Ready to use as supplied.

### 5.8 Human Neutrophil Elastase Assay Reagent (18 U/ml)

1. To use the enzyme as a positive control, add 2  $\mu$ L to 2 mL of NET Assay Buffer. Mix well. Add 100  $\mu$ L of this diluted enzyme into at least two wells in the assay plate.
2. To use the enzyme to produce a standard curve, please see section 7.

## **5.9 NET Assay Neutrophil Elastase Substrate**

1. To assay 24 samples in duplicate and a standard curve, dilute 225  $\mu$ L Substrate into 6.5 mL PBS (1:30 dilution).

## **5.10 S7 Nuclease Assay Reagent**

1. For one 24 well plate, dilute 12  $\mu$ L of S7 Nuclease (supplied at 15,000 U/mL) in 12 mL of pre-warmed NET Assay Buffer immediately prior to use to make a 15 U/mL working solution.



## 6. Standard Preparation

- Always prepare a fresh set of standards for every use.
  - Discard working standard dilutions after use as they do not store well.
1. While NET Assay Buffer will serve as an adequate diluent for the Human Neutrophil Assay Reagent, we recommend adding 400  $\mu$ L of EDTA Assay Reagent to 20 mL of NET Assay Buffer and using this for the dilutions of the Human Neutrophil Assay Reagent.
  2. Label eight clean test tubes #1 - #8.
  3. Add 5 mL of NET Assay Buffer into tube #1 and 1 mL into tubes #2 - #8.
  4. Transfer 10  $\mu$ L of Human Neutrophil Elastase Reagent into tube #1 and mix thoroughly. The concentration of this standard is 36 mU/mL.
  5. Serially dilute the standard by removing 1 mL from tube #1 and placing it into tube #2; mix thoroughly.
  6. Repeat for tubes #2 - #7.
  7. Do not add to tube #8 as this will be the blank.
  8. Dilution range will be 36, 18, 9, 4.5, 2.25, 1.125, 0.56, and 0 mU/well.

## 7. Sample Preparation

### General sample information:

We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.

We recommend that you use fresh samples for the most reproducible assay.

The following is designed for a 24-well plate (not provided). For other sizes of plate, the volume of medium/solution to apply to each well should be adjusted accordingly

### 7.1 Cell treatment:

1. Suspend NET-forming cells (e.g., human peripheral blood neutrophils) in pre-warmed NET Assay Buffer. We recommend a concentration of at least  $1 \times 10^6$  cells/mL. Add 900  $\mu$ L of cells per well. Be sure to include two wells containing culture medium only for background controls.
2. Treat cells with 100  $\mu$ L of the 10X working stock of PMA or A-23187. Incubate at 37°C for 4 hours, or for the time used in your typical experimental protocol to induce NET.
3. After stimulation and NET formation are complete, gently aspirate the NET Assay Buffer from the wells and slowly add 1 mL of pre-warmed NET Assay Buffer to the sides of the wells. Repeat for a total of two 1 mL washes. This removes soluble neutrophil elastase that is not NET-associated.
4. Add 500  $\mu$ L of the diluted (1:1000) S7 Nuclease to each well. Incubate for 15 minutes at 37°C to disrupt the NETs.  
 **$\Delta$ Note:** For higher cell concentrations, longer incubations (up to one hour) or more S7 Nuclease (up to 100 U/mL) may be required.
5. Transfer the supernatants to polypropylene microfuge tubes. Add 10  $\mu$ L of EDTA (500 mM) Assay Reagent solution to inactivate the nuclease. Centrifuge at 300 x *g* for 5 minutes to pellet any cellular debris.
6. Transfer supernatant to a new polypropylene tube or other appropriate storage container. Assay for released neutrophil elastase immediately. Or store at +4°C for one week or -20°C for up to 6 months before performing the neutrophil elastase assay.

## 8. Assay Procedure

- Equilibrate all materials and prepared reagents to 37°C in a water-bath prior to use.
- Assay all standards, controls and samples in duplicate.

**ΔNote:** If you suspect your samples contain substance that can generate background, set up Sample Background Controls to correct for background noise.

### 8.1 Performing the Elastase Activity Assay:

1. For standard wells add 100  $\mu$ L standard dilutions from tubes #1 - #8.
2. For sample wells transfer 100  $\mu$ L of culture supernatant per well (prepared in section 8).
3. Add 100  $\mu$ L of the 1:30 diluted NET Assay Neutrophil Elastase Substrate to each well.
4. Cover the plate with the 96-well cover sheet and incubate the plate for 1-2 hours at 37°C.
5. Remove the cover sheet and read the absorbance at 405 nm.

## 9. Data Analysis

Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

1. Average the duplicate reading for each standard, control and sample.
2. Subtract the mean value of the blank (Standard #8) from all standards, controls and sample readings. This is the corrected absorbance.
3. If significant, subtract the sample background control from sample readings.
4. Plot absorbance (linear y-axis) versus concentration (linear x-axis) for standards #1 - #8 and fit the data with a quadratic equation.
5. Using the equation of the line calculate the elastase activity in each sample.
6. Alternatively, a plot of concentration (y-axis) and absorbance (x-axis) can be performed. This plot has the benefit of easier calculation of elastase activity based on the best fit quadratic equation.

## 10.FAQs / Troubleshooting

Problem	Possible Causes	Recommended Solutions
Poor NET formation	NET assay buffer not pre-warmed to 37°C	Incubate longer than 4 hours
Inadequate NET release/disintegration	No calcium in assay buffer	Add calcium chloride to 1 mM
High level of elastase in control samples	Incomplete washing and removal of soluble elastase	Wash more thoroughly

## 11. Typical Data

Data provided for demonstration purposes only.

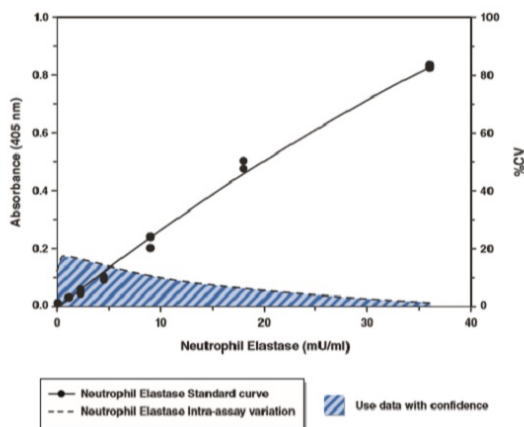
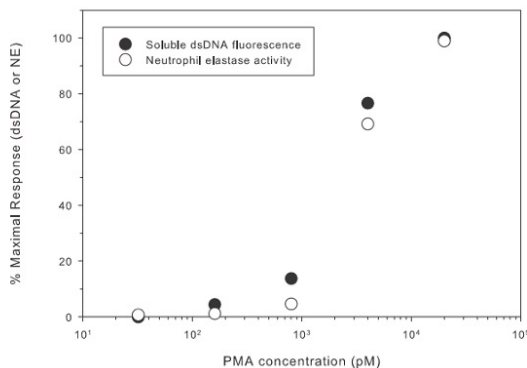


Figure 1. Human neutrophil elastase standard curve.



**Figure 2.** Measurement of released neutrophil elastase parallels measurement of released dsDNA. Human neutrophils were treated with PMA for 4 hours, washed and treated with S7 Nuclease for 15 minutes. The supernatant from each well was sampled and assayed for neutrophil elastase according to the protocol booklet. The supernatant was also tested for the presence of soluble dsDNA using a green fluorescence probe.

## 12. Notes

# Technical Support

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