NETosis Assay Kit

Item No. 601010

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Customer Service 800.364.9897
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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
400145	PMA (1 mM) Assay Reagent	1 vial/50 µl	-20°C
601011	S7 Nuclease Assay Reagent	1 vial/50 µl	-20°C
601012	EDTA (500 mM) Assay Reagent	1 vial/1 ml	RT
601013	NET Assay Neutrophil Elastase Substrate	1 vial/250 µl	-20°C
601014	Human Neutrophil Elastase Assay Reagent	1 vial/50 µl	-20°C
400085	A-23187 (25 mM) Assay Reagent	1 vial/50 µl	-20°C
400086	Bovine Serum Albumin Assay Reagent	1 vial/5 g	4°C
400087	Calcium Chloride (1 M) Assay Reagent	1 vial/1 ml	RT
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate	RT
400012	96-Well Cover Sheet	1 cover	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section on page 3 and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. RPMI cell culture medium
2. A source of NET-producing cells (*e.g.*, human peripheral blood neutrophils)
3. A 24-well tissue culture plate
4. A plate reader with the capacity to measure absorbance at 400-420 nm
5. A source of pure water; glass distilled water or HPLC-grade water is acceptable
6. Phosphate Buffered Saline (PBS)

About This Assay

Cayman's NETosis Assay Kit provides a simple and fast method for studying the process of NETosis *ex vivo*. Notably, Cayman's NETosis Assay Kit does not depend upon the DNA component of NETs, as DNA release can occur independently of NETosis. In this kit, primary neutrophils are stimulated to release NETs with either PMA or a calcium ionophore (both included). As shown in Figure 1, 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.

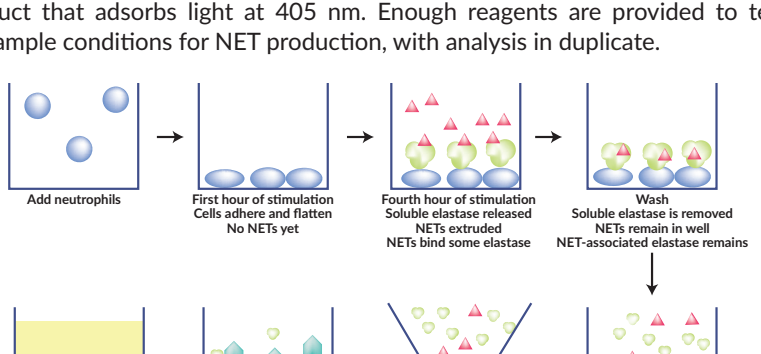

Figure 1. Step-by-step diagram of NET formation and analysis.

Reagent Preparation

1. **NET Assay Buffer (basal medium not included in kit)**

   To prepare the NET Assay Buffer, combine 500 ml of RPMI 1640 base medium (not provided) with 5 g Bovine Serum Albumin (Item No. 400086) and 500 µl of 1M Calcium Chloride (Item No. 400087). 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. *NOTE: Serum contains DNAse that will digest NETs and should be avoided if possible.* **Pre-warm to 37°C** prior to cell stimulation and addition of nuclease to ensure rapid activation and subsequent nuclease activity.

2. **PMA (1 mM) Assay Reagent - (Item No. 400145)**

   Prior to use, add 1 µl of 1 mM PMA to 5 ml of pre-warmed NET Assay Buffer to make a 10X working stock. PMA is a potential carcinogen. Wear gloves when using this reagent.

3. **A-23187 (25 mM) Assay Reagent - (Item No. 400085)**

   Prior to use, add 10 µl of 25 mM A-23187 to 1 ml of pre-warmed NET Assay Buffer to make a 10X working stock.

4. **S7 Nuclease Assay Reagent (Item No. 601011)**

   For one 24 well plate, dilute 12 µl 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.

5. **NET Assay Neutrophil Elastase Substrate (Item No. 601013)**

   The vial contains 15 mM N-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide as a substrate for neutrophil elastase. To assay 24 samples in duplicate and a standard curve, dilute 225 µl Substrate into 6.5 ml PBS (a 1:30 dilution).

6. **Human Neutrophil Elastase Assay Reagent (Item 601014)**

   This vial contains human neutrophil elastase at 18 U/ml. To use the enzyme as a positive control, add 2 µl to 2 ml of NET Assay Buffer. Mix well. Add 100 µl of this diluted Enzyme into at least two wells in the assay plate.

   To run a standard curve using the Human Neutrophil Elastase Assay Reagent, obtain eight clean test tubes and label them #1 through #8. *NOTE: While NET Assay Buffer will serve as an adequate diluent for the Human Neutrophil Elastase Assay Reagent, we recommend adding 400 µl of EDTA (500 mM) Assay Reagent (Item No. 601012) to 20 ml of NET Assay Buffer and using this for the dilutions of the Human Neutrophil Elastase Assay Reagent.* Add 5 ml of NET Assay Buffer into tubes #1 and 1 ml into #2-8. Transfer 10 µl of Human Neutrophil Elastase Assay Reagent into tube #1 and mix thoroughly. The concentration of this standard is 36 mU/ml. Serially dilute the standard by removing 1 ml from tube #1 and placing it into tube #2; mix thoroughly. Next remove 1 ml from tube #2 and place it into tube #3; mix thoroughly. Repeat for tubes #4-7. Do not add any standard to tube #8. This tube will be your blank.

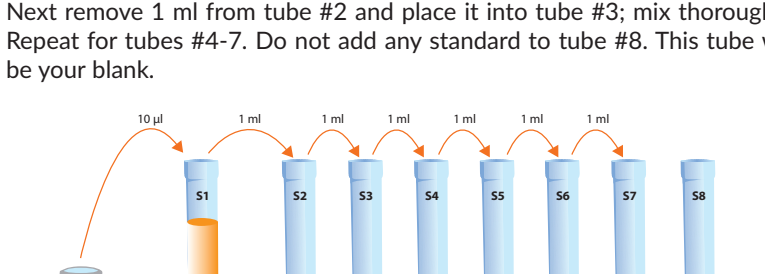

Figure 2. Preparation of the positive controls

Sample Preparation

Treatment of Cells

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

1. Suspend NET-forming cells (*e.g.*, human peripheral blood neutrophils) in pre-warmed NET Assay Buffer. We recommended a concentration of at least 1 x 10^6 cells/ml. Add 900 µl of cells per well. Be sure to include two wells containing culture medium only for background controls.
2. Treat the cells with 100 µl of the 10X working stock of PMA or A-23187. Incubate at 37°C for four hours, or for the period of time used in your typical experimental protocol to induce NET formation.
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 µl of the diluted (1:1,000) S7 Nuclease to each well. Incubate 15 minutes at 37°C to disrupt the NETs. *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 µl of EDTA (500 mM) Assay Reagent solution (Item No. 601012, provided at 500 mM) to inactivate the nuclease. Centrifuge at 300 x g for five 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 six months before performing the neutrophil elastase assay.

ASSAY PROTOCOL

Performing the Elastase Activity Assay

There is no specific pattern for using the wells on the plate. Each standard and sample should be assayed at least in duplicate.

Use the clear 96-well assay plates included in the kit to perform the assay described below. For optimal results, we recommend pre-warming the standards and samples to 37°C in a water bath prior to performing the NETosis assay.

1. **Standard Wells -** add 100 µl of standard (tubes #1-8) per well.
2. **Sample Wells -** Transfer 100 µl of culture supernatant per well.
3. **Addition of the Elastase Substrate -** add 100 µl of the 1:30 diluted NET Assay Neutrophil Elastase Substrate to each well.
4. Cover the plate with the 96-well cover sheet (Item No. 400012) and incubate the plate for 1-2 hours at 37°C.
5. Remove the cover sheet and read the absorbance at 405 nm.

ANALYSIS

Calculations

Plotting the Standard Curve and Determining the Sample Elastase Activity:

Plot absorbance (linear y-axis) *versus* concentration (linear x-axis) for standards (S1-S8) and fit the data with a quadratic equation. Using the equation of the line, calculate the elastase activity in each sample. *Alternatively, a plot of concentration (y-axis) and absorbance (x-asis) can be performed. This plot has the benefit of easier calculation of elastase activity based on the best fit quadratic equation.*

Performance Characteristics

Sample Data:

The standard curve presented here is an example of the data typically produced using the assay protocol described above. However, your results will not be identical to these. You must run a new standard curve with each experiment.

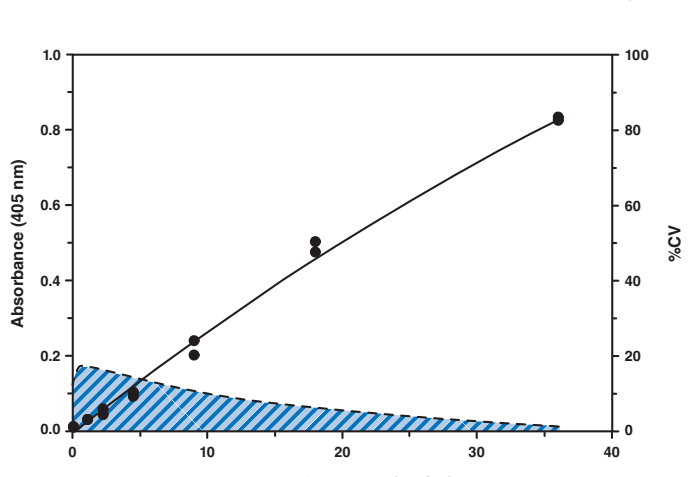

Figure 3. Human neutrophil elastase standard curve

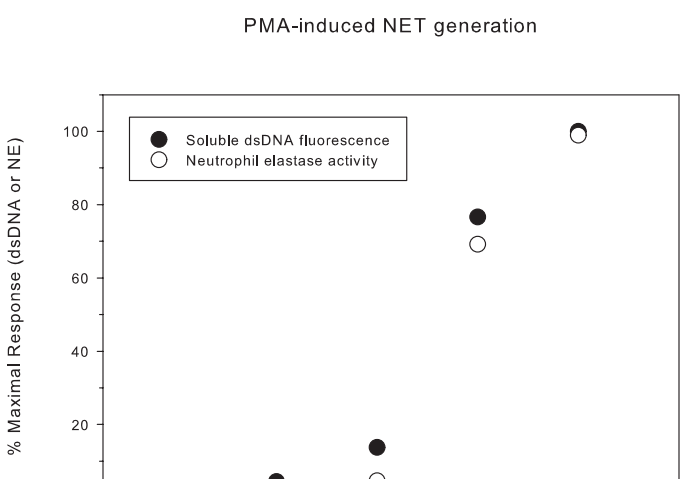

Figure 4. Measurement of released neutrophil elastase parallels measurement of released dsDNA.
Human neutrophils were treated with PMA for four hours, washed, and treated with S7 Nuclease for 15 minutes. The supernatant from each well was sampled and assayed for neutrophil elastase according to the procedure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

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Poor NET formation	NET assay buffer not pre-warmed to 37°C	Incubate longer than four 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

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.