NETosis Assay Kit

Item No. 601010

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Customer Service 800.364.9897
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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. After opening the kit, store individual components 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	2 vials/250 µl	-20°C
601014	Human Neutrophil Elastase Assay Reagent	1 vial/50 µl	-20°C
400085	A23187 (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 ea	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.

This kit may not perform as described if any reagent or procedure is replaced or modified.

PMA is a potential carcinogen. Please wear appropriate personal protection equipment (*e.g.* safety glasses, gloves, and lab coat) when using this material.

If You Have Problems

Technical Service Contact Information

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

Email: techserv@caymanchem.com

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 OR 96-well tissue culture plate
4. A plate reader with the capacity to measure absorbance at 400-420 nm
5. Phosphate-buffered saline (PBS)

INTRODUCTION

Background

Neutrophil extracellular traps (NETs) are an important component of the innate immune system, and NETosis is the process by which they are released.[1,2] NETs are composed of DNA, histones, neutrophil elastase, myeloperoxidase, and other microbicidal proteins.[2] NETs can trap and immobilize pathogens and also have direct antimicrobial activity. The process of NETosis occurs over a period of hours and can lead to a lytic form of cell death that differs from apoptosis and necrosis.[3,4] Interestingly, neutrophils may expel NETs without induction of cell lysis, becoming cytoplasts or "neutrophil ghosts", while preserving chemotactic and phagocytic functions.[3] Aberrant NETosis or defects in NET clearance have been implicated in a host of inflammatory disorders and autoimmune diseases, including systemic lupus erythematosus (SLE), psoriasis, and rheumatoid arthritis.[5,6]

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 digestion of NET DNA by S7 nuclease, the supernatant containing neutrophil elastase is added to a substrate, which is selectively cleaved by neutrophil elastase to yield a 4-nitroaniline product that absorbs light at 405 nm. A sufficient amount of each reagent is provided to test and analyze up to 80 individual samples of NETosis stimulators or inhibitors or 24 samples in duplicate.

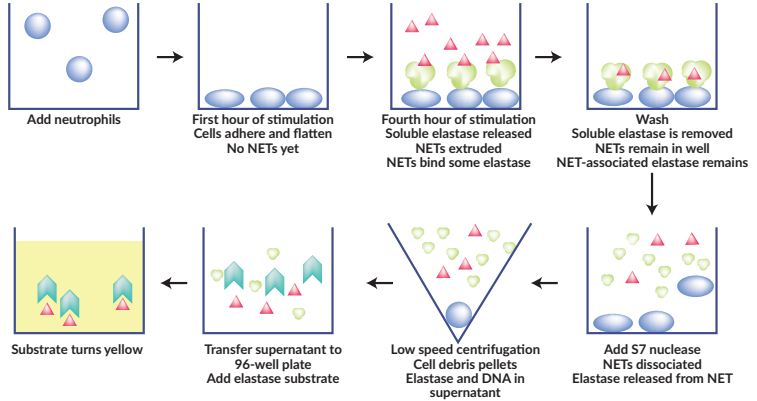

Figure 1. Step-by-step diagram of NET formation and analysis

PRE-ASSAY PREPARATION FOR 24-WELL ASSAY

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 cell culture medium (not provided) with 5 g of Bovine Serum Albumin Assay Reagent (Item No. 400086) and 500 µl of Calcium Chloride (1 M) Assay Reagent (Item No. 400087). The NET assay buffer is not intended to be sterile and does not need to be prepared or used in a tissue culture hood. Pre-warm the NET assay buffer to 37°C prior to cell stimulation and addition of nuclease to ensure rapid activation and subsequent nuclease activity. 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.*

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

   Prior to use, add 1 µl of PMA (1 mM) Assay Reagent to 5 ml of pre-warmed NET assay buffer to make a 10X working stock solution. *NOTE: PMA is a potential carcinogen. Wear appropriate protection and use caution when handling this solution.*

3. **A23187 (25 mM) Assay Reagent - (Item No. 400085)**

   Prior to use, add 10 µl of A23187 (25 mM) Assay Reagent to 1 ml of pre-warmed NET assay buffer to make a 10X working stock solution.

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

   For one 24-well plate, dilute 12 µl of S7 Nuclease Assay Reagent (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 run a standard curve, dilute 225 µl NET Assay Neutrophil Elastase Substrate into 6.5 ml PBS (a 1:30 dilution).

6. **Human Neutrophil Elastase Assay Reagent (Item No. 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 pre-warmed NET assay buffer. Mix well. Add 100 µl of this diluted enzyme into at least two wells of 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 the 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 pre-warmed NET assay buffer into tube #1 and 1 ml into tubes #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.

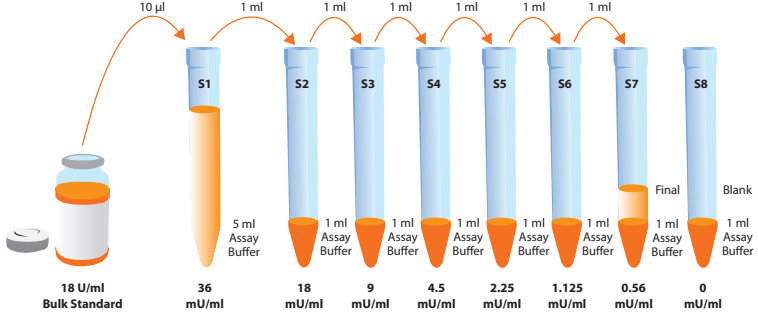

Figure 2. Preparation of the positive controls

Sample Preparation

Treatment of Cells

The following protocol is designed for a 24-well tissue culture plate (not provided).

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 800 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 solution of PMA or A23187. 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 Assay Reagent to each well. Incubate at 37°C for 15 minutes 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 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.

PRE-ASSAY PREPARATION FOR 96-WELL ASSAY

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 of Bovine Serum Albumin Assay Reagent (Item No. 400086) and 500 µl of Calcium Chloride (1 M) Assay Reagent (Item No. 400087). The NET assay buffer is not intended to be sterile and does not need to be prepared or used in a tissue culture hood. Pre-warm the NET assay buffer to 37°C prior to cell stimulation and addition of nuclease to ensure rapid activation and subsequent nuclease activity. 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.*

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

   Prior to use, add 2 µl of PMA (1 mM) Assay Reagent to 1 ml of NET assay buffer to make a 2 µM intermediate stock solution. Addition of 40 µl of this intermediate stock solution to 10 ml of pre-warmed NET assay buffer will make a 4X working stock solution. The final in-well concentration using this dilution scheme is 1 nM, but concentrations up to 100 nM are acceptable and commonly used. *NOTE: PMA is a potential carcinogen. Wear appropriate protection and use caution when handling this solution.*

3. **A23187 (25 mM) Assay Reagent - (Item No. 400085)**

   Prior to use, add 20 µl of A23187 (25 mM) Assay Reagent to 5 ml of pre-warmed NET assay buffer to make a 4X working stock solution.

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

   For one 96-well plate, dilute 50 µl of S7 Nuclease Assay Reagent (supplied at 15,000 U/ml) in 20 ml of pre-warmed NET assay buffer immediately prior to use to make a 37.5 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 80 samples and run a standard curve, dilute 500 µl NET Assay Neutrophil Elastase Substrate into 14.5 ml PBS (a 1:30 dilution).

6. **Human Neutrophil Elastase Assay Reagent (Item No. 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 pre-warmed NET assay buffer. Mix well. Add 100 µl of this diluted enzyme into at least two wells of 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 the 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 pre-warmed NET assay buffer into tube #1 and 1 ml into tubes #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.

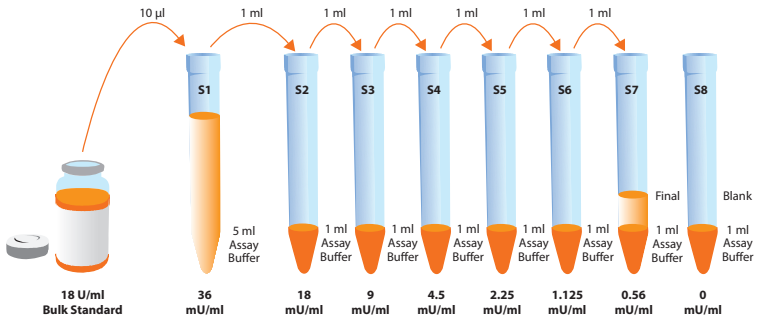

Figure 3. Preparation of the positive controls

Sample Preparation

Treatment of Cells

The following protocol is designed for a 96-well tissue culture plate (not provided). *NOTE: The neutrophil elastase standard curve will utilize 16 wells of the plate, so it is recommended to only use 80 wells for this assay.*

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 x 10^6 cells/ml. Add 100 µl of cells per well. Be sure to include at least two wells containing culture medium only for background controls.
2. **Add NETosis stimuli:** add 50 µl of the 4X working stock solution of PMA or A23187, 50 µl of 4X test NETosis stimuli, or 50 µl of NET assay buffer to each well, for a volume of 150 µl.
3. **Add NETosis inhibitors:** add 50 µl of test compounds prepared at 4X and 50 µl of vehicle prepared in NET assay buffer to the appropriate wells. Add 50 µl NET assay buffer to the remaining wells such that the final volume in all wells is 200 µl. Incubate at 37°C for four hours.
4. After stimulation and NET formation are complete, gently aspirate the NET assay buffer from the wells and slowly add 100 µl of pre-warmed NET assay buffer to the sides of the wells. Repeat for a total of two 100 µl washes. This removes soluble neutrophil elastase that is not NET-associated.
5. Add 200 µl of the diluted (37.5 U/ml) S7 Nuclease Assay Reagent to each well. Incubate at 37°C for 45 minutes 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.*
6. Add 2 µl of EDTA (500 mM) Assay Reagent to each well to inactivate the nuclease. Centrifuge at 300 x g for five minutes to pellet any cellular debris.
7. Transfer supernatant to a separate 96-well tissue culture plate 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.

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 plate 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.

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-axis) 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.

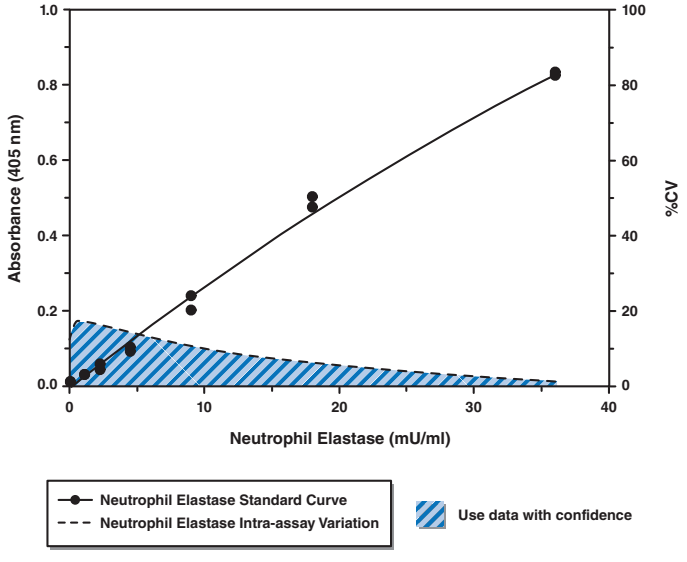

Figure 4. Human neutrophil elastase standard curve

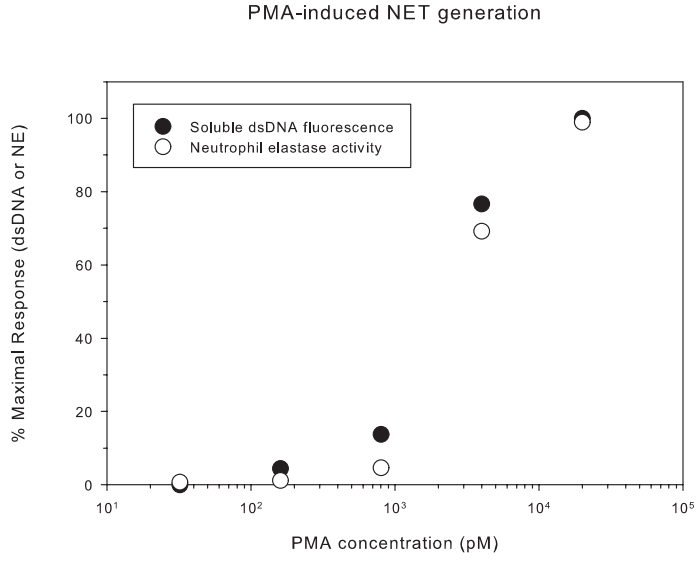

Figure 5. Measurement of released neutrophil elastase parallels measurement of released dsDNA. Human neutrophils were treated with PMA for four hours, washed, and treated with S7 nuclease for 15 minutes. The supernatant from each well was sampled and assayed for neutrophil elastase according to the procedure described in the kit booklet. The supernatant was also tested for the presence of soluble dsDNA using PicoGreen fluorescence.

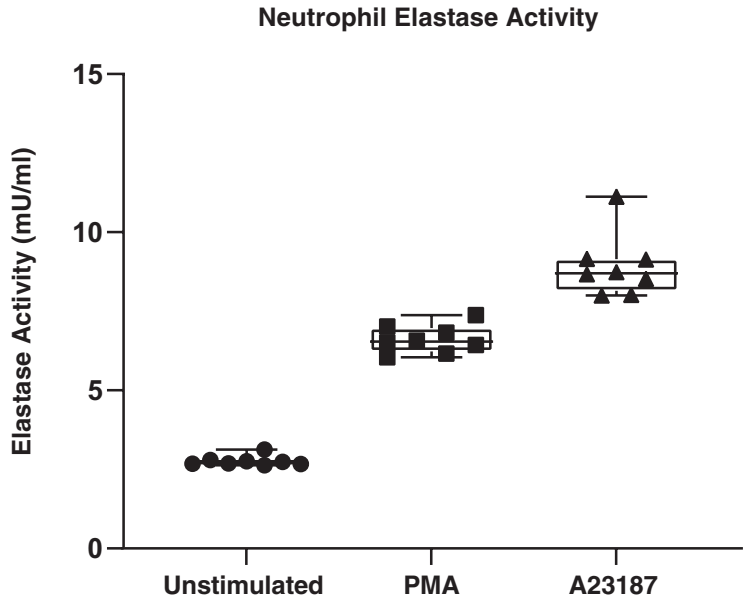

Figure 6. Measurement of released neutrophil elastase following stimulation. Human neutrophils were seeded in a 96-well plate and treated with PMA or A23187 for four hours as described above. After stimulation, cells were washed and treated with S7 nuclease for 45 minutes. The supernatant from each well was sampled and assayed for neutrophil elastase activity according to the procedures described in the kit booklet.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Poor NET formation	A. Incubation was not long enough B. NET assay buffer was not pre-warmed to 37°C	A. Incubate longer than four hours B. Pre-warm NET assay buffer
Inadequate NET release/disintegration	No calcium in NET assay buffer	Add calcium chloride to NET assay buffer at a concentration of 1 mM
High level of elastase in control samples	Incomplete washing and removal of soluble elastase	Wash more thoroughly

References

1. Brinkmann, V., Reichard, U., Goosmann, C., *et al.* Neutrophil extracellular traps kill bacteria. *Science* **303(5663)**, 1532-1535 (2004).
2. Dabrowska, D., Jablonska, E., Garley, M., *et al.* New aspects of the biology of neutrophil extracellular traps. *Scand. J. Immunol.* **84(6)**, 317-322 (2016).
3. Yipp, B.G. and Kubes, P. NETosis: How vital is it? *Blood* **122(16)**, 2784-2794 (2013).
4. Gupta, S., Chan, D.W., Zaal, K.J., *et al.* A high-throughput real-time imaging technique to quantify NETosis and distinguish mechanisms of cell death in human neutrophils. *J. Immunol.* **200(2)**, 869-879 (2017).
5. Vorobjeva, N.V. and Pinegin, B.V. Neutrophil extracellular traps: Mechanisms of formation and role in health and disease. *Biochemistry (Mosc.)* **79(12)**, 1286-1296 (2014).
6. Pinegin, B., Vorobjeva, N.V., and Pinegin, V. Neutrophil extracellular traps and their role in the development of chronic inflammation and autoimmunity. *Autoimmun. Rev.* **14(7)**, 633-640 (2015).

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.