Store at -20°C

NETosis Assay Kit

#41855

1 Kit
(96 assays)

Cell Signaling TECHNOLOGY®

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For Research Use Only. Not for Use in Diagnostic Procedures.

Product Includes	Product #	Kit Quantity	Storage Temp
PMA (1 mM) Assay Reagent	51640	1 x 50 µL	-20°C
S7 Nuclease Assay Reagent	60484	1 x 50 µL	-20°C
NET Assay Neutrophil Elastase Substrate	34136	2 x 250 µL	-20°C
Human Neutrophil Elastase Assay Reagent	42211	1 x 50 µL	-20°C
Bovine Serum Albumin Assay Reagent	50855	1 x 5 g	4°C
EDTA (500 mM) Assay Reagent	79598	1 x 1 mL	RT
Calcium Chloride (1 M) Assay Reagent	60648	1 x 1 mL	RT
96-Well Solid Plate (Colorimetric Assay)	75820	96 tests	RT
96-Well Cover Sheet	87187	1ea	RT

Description: The NETosis Assay Kit is a quantitative colorimetric assay that can be used to study the process of NETosis. In this kit, primary neutrophils are stimulated with PMA (included in the kit) to release neutrophil extracellular traps (NETs), leading to the production of neutrophil elastase. The unbound neutrophil elastase will be washed away, leaving behind NETs with bound neutrophil elastase. The S7 nuclease is used to digest NET DNA, releasing the neutrophil elastase from the NETs. When the neutrophil elastase substrate is added to the samples, the substrate is selectively cleaved by the neutrophil elastase to produce 4-nitroaniline that absorbs light at 405 nm. The NETosis Assay Kit does not depend upon the DNA component of NETs, as DNA release can occur independently of NETosis. The kit provides enough reagents to test up to 80 individual samples with 16 standard curve wells in a 96-well plate, or 24 samples in duplicate in a 24-well plate.

Background: NETosis is a unique form of regulated cell death that is characterized by membrane rupture and the extrusion of chromatin, histones, and granular and cytoplasmic components into a web-like structure called neutrophil extracellular traps (NETs) (reviewed in 1). NETosis has been associated with host defense to pathogens as well as a number of disease states, including autoimmune diseases, thrombosis, cardiovascular diseases, and tumor progression. NETosis was identified as a response to bacterial infection and can be activated by lipopolysaccharide (LPS) as well as inflammatory pathway activators like phorbol-12-myristate-13-acetate (PMA) (2). It can occur via multiple pathways, but several key players have emerged. The calcium-dependent enzyme protein-arginine deiminase 4 (PAD4) catalyzes hypercitrullination of histones that contributes to chromatin decondensation (3,4). In addition, activation of proteases, including neutrophil elastase (ELANE), myeloperoxidase (MPO), and Cathepsin G, leads to impairment of cytoskeletal structures and degradation of histones during NETosis (5,6).

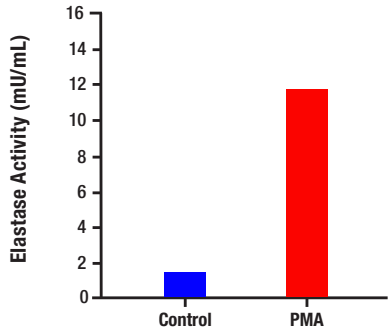

Figure 1. Determination of released neutrophil elastase activity following PMA stimulation. *Human neutrophils were seeded in a 96-well (or 24-well) plate and treated with PMA for four hr as described above. Following stimulation, cells were washed and treated with S7 nuclease for 45 min. Supernatant from each well was assayed and neutrophil elastase activity was determined as described. Bar graphs represent the average elastase activity from three independent neutrophil donors and error bars depict the standard deviation.*

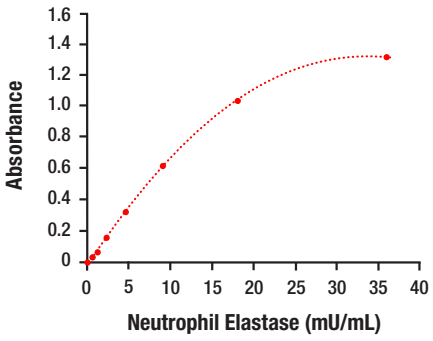

Figure 2*. Human neutrophil elastase standard curve data is shown.*

Storage: All components in this kit are stable for at least 12 months when stored at the recommended temperature.

Please visit cellsignal.com for validation data and a complete listing of recommended companion products.

Background References:

(1) Thiam, H.R. et al. (2020) *Annu Rev Cell Dev Biol* 36, 191-218.
(2) Brinkmann, V. et al. (2004) *Science* 303, 1532-5.
(3) Li, P. et al. (2010) *J Exp Med 207*, 1853-62.
(4) Wong, S.L. and Wagner, D.D. (2018) *FASEB J*, fj201800691R.
(5) Papayannopoulos, V. et al. (2010) *J Cell Biol* 191, 677-91.
(6) Metzler, K.D. et al. (2014) *Cell Rep* 8, 883-96.

Applications: W—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **FC-FP**— Flow cytometry-Fixed/Permeabilized **FC-L**— Flow cytometry-Live **E-P**—ELISA-Peptide
Species Cross-Reactivity: H—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **Dm**—D. melanogaster **X**—Xenopus **Z**—zebrafish **B**—bovine **Dg**—dog **Pg**—pig **Sc**—S. cerevisiae **Ce**—C. elegans **Hr**—Horse
All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

NETosis Assay Kit Protocol

Solutions and Reagents

Supplied Reagents

1. **PMA (1 mM) Assay Reagent:** #51640
2. **S7 Nuclease Assay Reagent:** #60484
3. **EDTA (500 mM) Assay Reagent:** #79598
4. **NET Assay Neutrophil Elastase Substrate (#34136):** The vial contains 15 mM N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide as a substrate for neutrophil elastase.
5. **Human Neutrophil Elastase Assay Reagent (#42211):** The 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.
6. **Bovine Serum Albumin Assay Reagent:** #50855
7. **Calcium Chloride (1 M) Assay Reagent:** #60648
8. **96-Well Solid Plate (Colorimetric Assay):** #75820
9. **96-Well Cover Sheet:** #87187

NET Assay Buffer (basal medium not included in the 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 #50855 and 500 µL of Calcium Chloride (1 M) Assay Reagent #60648. 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.

Standard Curve

To run a standard curve using the Human Neutrophil Elastase Assay Reagent #42211, 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 (#79598) 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.

Reagent Preparation for treating cells in a 96-well plate (not provided)

1. **PMA (1 mM) Assay Reagent #51640** - Prior to use, add 2 µL of PMA (1 mM) Assay Reagent to 1 mL of pre-warmed NET assay buffer to make a 2 µM intermediate stock solution. Subsequently, 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.

   NOTE: PMA is a potential carcinogen. Wear appropriate protection and use caution when handling this solution.
2. **S7 Nuclease Assay Reagent #60484** - 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.
3. **NET Assay Neutrophil Elastase Substrate #34136** - 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).

Reagent Preparation for treating cells in a 24-well plate (not provided)

1. PMA (1 mM) Assay Reagent #51640 - 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.
2. S7 Nuclease Assay Reagent #60484 - 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.
3. NET Assay Neutrophil Elastase Substrate #34136 - 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).

Additional Reagents (Not Supplied)

1. RPMI cell culture medium
2. A source of NET-producing cells (e.g., human peripheral blood neutrophils)
3. A 96-well or 24-well tissue culture plate
4. A plate reader with the capacity to measure absorbance at 400-420 nm
5. Phosphate-buffered saline (PBS)

NETosis Assay Kit Protocol

Treating Cells

The following protocol is designed for a 96-well or 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 recommend a concentration of at least 1 x 10^6 cells/mL. Add 100 µL of cells per well (96-well) or 800 µL of cells per well (24-well). Be sure to include two wells containing culture medium only for background controls.
2. If working with a 96-well plate, add 50 µL of the 4X working stock solution of PMA or 50 µL of blank NET assay buffer to each well. If working with a 24-well plate, add 100 µL of the 10X working stock solution of PMA or 100 µL of blank NET assay buffer to each well. Incubate at 37°C for four hr.
3. After stimulation and NET formation are complete, gently aspirate the NET assay buffer from the wells and slowly add 100 µL (96-well) or 1 mL (24-well) of pre-warmed NET assay buffer to the sides of the wells. Repeat for a total of two washes. This removes soluble neutrophil elastase that is not NET-associated.
4. If working with a 96-well plate, add 200 µL of the diluted (1:400) S7 Nuclease Assay Reagent to each well. Incubate at 37°C for 45 min to disrupt the NETs. If working with a 24-well plate, add 500 µL of the diluted (1:1000) S7 Nuclease Assay Reagent to each well. Incubate at 37°C for 15 min to disrupt the NETs.

   NOTE: For higher cell concentrations, longer incubations (up to one hr) or more S7 nuclease (up to 100 U/mL) may be required.
5. If working with a 96-well plate, add 2 µl of EDTA (500 mM) Assay Reagent to each well to inactivate the nuclease. If working with a 24-well plate, 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 min to pellet any cellular debris.
6. Transfer supernatant to a separate 96-well tissue culture plate, new polypropylene microfuge tubes, 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 Solid Plate (Colorimetric Assay) #75820 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 (#87187) and incubate the plate for 1-2 hr 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.