NETosis Imaging Assay Kit

Item No. 601750

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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
601077	RBC Lysis Buffer (10X)	1 vial/10 ml	4°C
600612	Cell-Based Assay Neutrophil Isolation Histopaque®	1 vial/25 ml	4°C
400145	PMA (1 mM) Assay Reagent	1 vial/50 µl	-20°C
400085	A23187 (25 mM) Assay Reagent	1 vial/50 µl	-20°C
400087	Calcium Chloride (1 M) Assay Reagent	1 vial/1 ml	RT
600198	NETosis Imaging Buffer (5X)	1 vial/25 ml	-20°C
600199	Extracellular Nuclear Green™ Reagent (5 mM)	1 vial/25 µl	-20°C
600239	Permeable Nuclear Red™ Reagent (5 mM)	1 vial/25 µl	-20°C
600243	Triton™ X-100 Permeabilization Control	1 vial/200 µl	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: 734-971-3640

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. Microscope or high-content imager capable of imaging at excitation/emission (ex/em) wavelengths of 503/526 and 622/645 nm (see table on page 7)
2. Heparin-collected fresh blood samples or a neutrophil-like cell line (*e.g.* DMSO-differentiated HL-60 cells). *NOTE: Other anticoagulants have not been verified in this assay. EDTA should be avoided.*
3. PBS, pH 7.4
4. A source of pure water; glass-distilled water or deionized water is acceptable. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*
5. 96-Well plate or other vessel suitable for imaging

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, alert other immune cells, such as macrophages, to clear these pathogens, and also have direct antimicrobial activity.[3] 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]

Traditional analyses of NETosis have relied on the quantification of NET components, such as DNA and neutrophil elastase, which are not exclusively released by NETting neutrophils. Alternative analyses use visualization of fixed cells in the process of NETosis. The limitation to these methods is that the parameters must be quantified at a single time point, making the kinetics of NETosis challenging to study. We have developed a simple staining protocol for use in high-content imaging systems to study the process of NETosis as it occurs.

About This Assay

Cayman's NETosis Imaging Kit provides a simple staining protocol for visualizing the process of NETosis kinetically *ex vivo* and *in vitro*. A cell-permeable DNA dye allows visualization of the dynamic changes of the nucleus during the process of NETosis, while a cell-impermeable dye detects the extruded DNA. Combining the fluorescence visualization of these dyes with brightfield imaging in a high-content platform allows for exquisite differentiation between different modes of cell death. The amount of each reagent provided is sufficient for two 96-well plates.

Dye	Excitation	Emission	Typical Filter	What it Stains
Extracellular Nuclear Green™ Reagent (5 mM)	503	526	GFP/FITC	Extracellular DNA/ permeabilized cells
Permeable Nuclear Red™ Reagent (5 mM)	622	645	Cy5	All nuclei

Table 1. Imaging parameters

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Cell-Based Assay Neutrophil Isolation Histopaque®**

   Bring Cell-Based Assay Neutrophil Isolation Histopaque® (Item No. 600612) to room temperature prior to use. Store at 4°C after use.

2. **Red Blood Cell (RBC) Lysis Buffer (1X)**

   Dilute 5 ml of RBC Lysis Buffer (10X) (Item No. 601077) with 45 ml room temperature pure water immediately before use. Scale as necessary, do not store 1X solution.

3. **NETosis Imaging Buffer (1X)**

   Dilute 10 ml of NETosis Imaging Buffer (5X) (Item No. 600198) with 40 ml pure water. Add 50 µl Calcium Chloride (1 M) Assay Reagent (Item No. 400087) for a final concentration of 1 mM. Unused buffer may be filter sterilized and stored at 4°C for up to 1 month.

4. **PMA Positive Control Stimulus (2X)**

   If required for your experimental design, dilute 5 µl PMA (1 mM) Assay Reagent (Item No. 400145) with 495 µl PBS to make a PMA Working Stock Solution (50X). Add 400 µl of the PMA Working Stock Solution (50X) to 9.6 ml NETosis Imaging Buffer (1X) to make sufficient PMA Positive Control Stimulus (2X) for one 96-well plate treated at a high concentration of 200 nM. Scale as necessary. Alternatively, a dose-response curve (in triplicate) can be generated by adding 40 µl of the PMA Working Stock Solution (50X) to 960 µl NETosis Imaging Buffer (1X) to make a high concentration of 200 nM. Half-log serial dilutions can then be prepared by serially diluting 316 µl of stock into 684 µl of NETosis Imaging Buffer (1X).

5. **A23187 Positive Control Stimulus (2X)**

   If required for your experimental design, dilute 20 µl A23187 (25 mM) Assay Reagent (Item No. 400085) in 10 ml NETosis Imaging Buffer (1X) to make sufficient A23187 Positive Control Stimulus (2X) for one 96-well plate treated at 25 µM. Alternatively, a dose-response curve (in triplicate) can be prepared by adding 4 µl A23187 to 996 µl NETosis Imaging Buffer (1X) to make a high concentration of 50 µM. Two-fold serial dilutions can then be prepared by serially diluting 500 µl of stock into 500 µl NETosis Imaging Buffer (1X).

ASSAY PROTOCOL

Performing the Assay

1. Collect 10-20 ml of blood into heparin blood collection tubes.
2. Transfer the blood to a 50 ml conical tube. Rinse blood collection tubes with 10 ml PBS and add to the conical tube.
3. Place 12 ml room temperature Cell-Based Assay Neutrophil Isolation Histopaque® (Item No. 600612) into a new conical tube. Carefully pipet diluted blood onto the top of the Cell-Based Assay Neutrophil Isolation Histopaque®.
4. Centrifuge at 500 x g for 20 minutes at room temperature, turning the centrifuge brake off, if possible.
5. Carefully aspirate the yellowish and clear upper layers, leaving the dark RBC and neutrophil layer.
6. Add 25 ml RBC Lysis Buffer (1X) and mix well.
7. Incubate at room temperature for 10 minutes.
8. Centrifuge at 250 x g for 5 minutes at room temperature to pellet the neutrophils.
9. Carefully aspirate the supernatant. *NOTE: If RBCs remain, add 10 ml RBC Lysis Buffer (1X) and incubate at room temperature for 2-5 minutes. Centrifuge at 250 x g for 5 minutes at room temperature and aspirate the supernatant.*
10. Resuspend the neutrophil pellet in 25 ml PBS and count the cells.
11. Remove 6 x 10^6 cells to a clean tube and adjust the concentration with PBS to 1 x 10^6 cells/ml. It may be necessary to centrifuge the cells at 250 x g, aspirate the supernatant, and resuspend in 6 ml PBS.
12. Add 6 µl Permeable Nuclear Red™ Reagent (5 mM) (Item No. 600239) and incubate in the dark at 37°C for 15-30 minutes.

    During this incubation prepare the positive control stimuli, as described in the Reagent Preparation Section (see page 8), and any other stimuli required by your experimental design.
13. Add 25 ml PBS to the cells, centrifuge at 250 x g, and aspirate the supernatant.
14. Resuspend the cells in 12 ml (0.5 x 10^6 cells/ml) NETosis Imaging Buffer (1X).
15. Add 12 µl Extracellular Nuclear Green™ Reagent (5 mM) (Item No. 600199) mix well and distribute 100 µl/well to a 96-well plate.
16. Add 100 µl of chosen stimuli or NETosis Imaging Buffer (1X) to the wells for a final volume of 200 µl.
17. Centrifuge plate briefly at 250 x g to collect cells at the bottom of the wells.
18. Image kinetically, using brightfield and fluorescence microscopy at the wavelengths recommended in the table on page 7, every 15-30 minutes for 6-12 hours, holding the temperature of the plate at 37°C.

    NOTE: If required for your instrument set-up, add 10 µl Triton™ X-100 Permeabilization Control (Item No. 600243) to 1-2 control wells. These wells can be used to set imaging parameters for Extracellular Nuclear Green™.

ANALYSIS

NETs can be identified as Extracellular Nuclear Green™-positive cells. Most image analysis software allows a threshold to be set for green intensity, and PMA- or A23187-stimulated cells at a late timepoint are a benchmark against which to set this. Assuming similar starting numbers of cells in each well and sufficient separation of cells, the number of NETs can be counted in each field of view or the total area per field of view can be calculated. The integral, or area under the curve (area *vs.* time), can also be used to quantify NET formation over time for a particular well. Qualitatively, cells undergoing NETosis can be observed, using both brightfield and Permeable Nuclear Red™ fluorescence, to swell and spread out (brightfield) and decondense their chromatin (red fluorescence).

Performance Characteristics

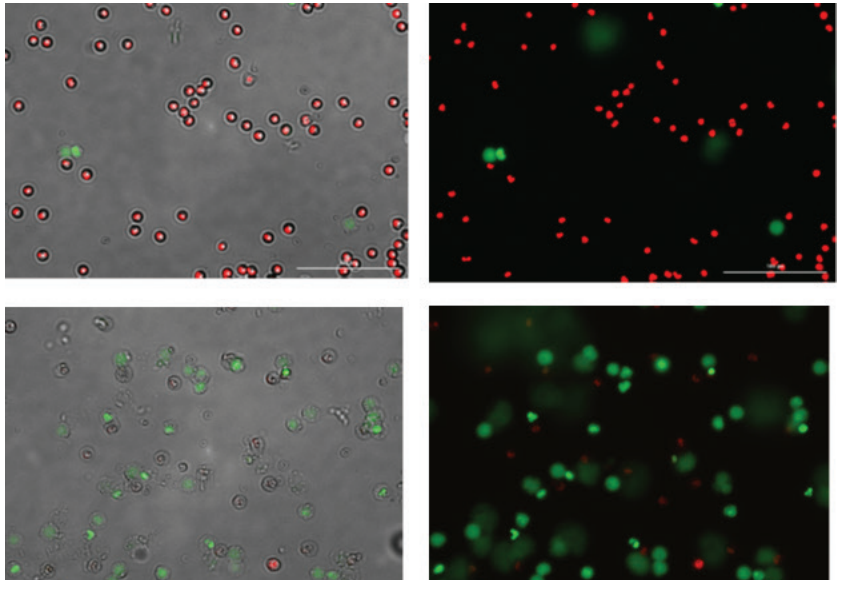

Figure 1. NETosis can be detected using the cell-impermeable DNA dye Extracellular Nuclear Green™ Primary blood neutrophils were isolated, stained with Extracellular Nuclear Green™ and Permeable Nuclear Red™, and left unstimulated (top) or stimulated (bottom) with 20 nM PMA for 3 hours. Images were generated using BioTek®'s Cytation™ 5 Cell Imaging Multi-Mode Reader with brightfield (left only) and GFP and Cy5 LED filter sets.

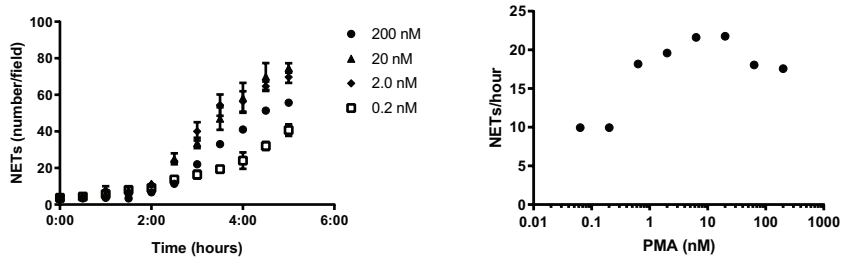

Figure 2. NETosis can be quantified by counting Extracellular Nuclear Green™-positive cells PMA-stimulated primary blood neutrophils were prepared and imaged as described in Figure 1. Cells positive for Extracellular Nuclear Green™ were counted in each field at each time point (left panel). The linear portion of the kinetic curve was used and an hourly rate calculated (right panel) for each PMA concentration.

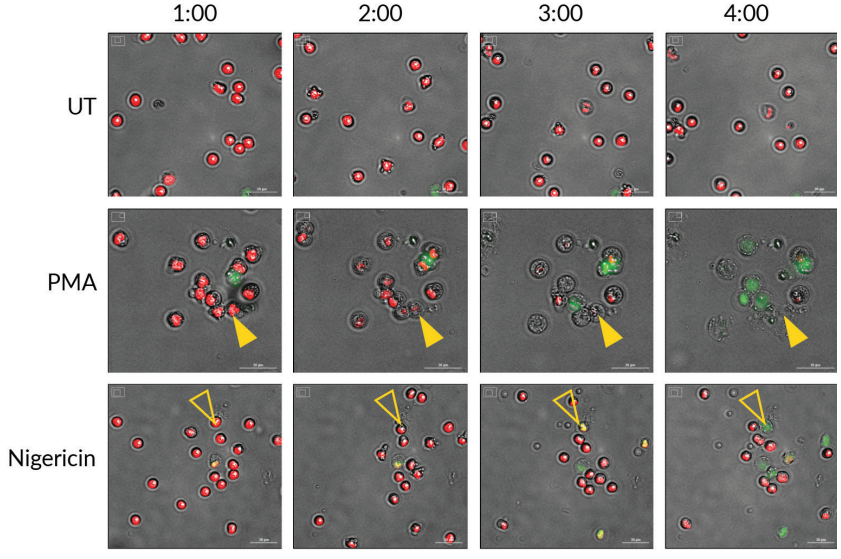

Figure 3. Monitoring the morphology of primary human neutrophils undergoing NETosis DNA extruded during the dynamic process of NETosis is detected by Extracellular Nuclear Green™ Reagent in neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA) or nigericin. Intact nuclei are counterstained with Permeable Nuclear Red™ Reagent. Timed images (every hour) were generated with BioTek®'s Cytation™ 5 Cell Imaging Multi-Mode Reader using a 20X objective and GFP and Cy5 LED filer combinations with brightfield. Scale bars are 30 µm, and filled and empty arrowheads follow individual cells undergoing NETosis.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Cells are green at the start of imaging	Neutrophils have compromised plasma membranes	Handle primary neutrophils gently and ensure all buffers are isotonic
No neutrophils appear to undergo NETosis	A. Wrong filters B. Cells are not neutrophilic C. Insufficient stimulus	A. Use filters shown in the table on page 7 B. Isolate primary neutrophils or confirm that the cell line differentiation protocol generates neutrophil-like cells C. Use positive control stimuli at suggested concentrations

References

1. Brinkmann, V., Reichard, U., Goosmann, C., *et al. Science* **303(5663)**, 1532-1535 (2004).
2. Dabrowska, D., Jablonska, E., Garley, M., *et al. Scand. J. Immunol.* **84(6)**, 317-322 (2016).
3. Yipp, B.G. and Kubes, P. *Blood* **122(16),** 2784-2794 (2013).
4. Gupta, S., Chan, D.W., Zaal, K.J., *et al. J. Immunol.* **200(2)**, 869-879 (2017).
5. Vorobjeva, N.V. and Pinegin, B.V. *Biochemistry (Mosc.)* **79(12)**, 1286-1296 (2014).
6. Pinegin, B., Vorobjeva, N.V., and Pinegin, V. *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.