NET Fragment Assay Kit (MPO-DNA)

Item No. 501330

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity	Storage
401144	Anti-MPO ELISA Strip Plate	1 plate	-20°C
401143	Anti-DNA HRP Conjugate (10X)	1 vial/1.5 ml	-20°C
400817	NET Fragment Control	1 vial/120 µl	-20°C
401146	Immunoassay Buffer F Concentrate (5X)	2 vials/10 ml	-20°C
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	RT
400035	Polysorbate 20	1 vial/3 ml	RT
400074	TMB Substrate Solution	1 vial/12 ml	4°C
10011355	HRP Stop Solution	1 vial/12 ml	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.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's NET Fragment Assay Kit (MPO-DNA). This kit may not perform as described if any reagent or procedure is replaced or modified.

The stop solution provided with this kit is an acid solution. 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 (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 450 nm
2. An orbital microplate shaker
3. Adjustable pipettes; multichannel or repeating pipettor recommended
4. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
5. Materials used for **Sample Preparation** (see page 11)

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 a web-like network of biomolecules composed of DNA, histones, neutrophil elastase, myeloperoxidase (MPO), and other microbicidal proteins that are released from neutrophils in response to pathogens.[2] MPO is a heme-containing peroxidase and the most abundant protein in polymorphonuclear leukocytes (PMNs).[3] It enhances neutrophil elastase-induced chromatin decondensation and produces reactive oxygen species (ROS), which trigger NET formation.[4] NETs can trap and immobilize pathogens and also have direct antimicrobial activity. The *in vitro* process of NETosis occurs over a period of hours and can lead to a lytic form of neutrophil cell death that differs from apoptosis and necrosis.[5,6] Interestingly, neutrophils may expel NETs without induction of cell lysis, becoming cytoplasts or "neutrophil ghosts", while preserving chemotactic and phagocytic functions.[5] Once released, NETs are cleared by plasma DNase and macrophages *via* phagocytosis of the NET fragments.[7] 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.[8,9] Increased levels of circulating NET fragments, containing MPO-DNA complexes, in serum have been associated with active small-vessel vasculitis, COVID-19, and cancer, highlighting the importance of continued research on NETs.[10-12]

About This Assay

Cayman's NET Fragment Assay Kit (MPO-DNA) is a qualitative assay that can be used for the detection of MPO-DNA complexes (*i.e.*, NET fragments) in human plasma and cell culture supernatants. The assay works as an immunometric (*i.e.*, sandwich) assay and requires intact MPO-DNA NET fragments for simultaneous binding to both the capture and detection antibodies. Interruption of these NET fragments by DNases or other intrinsic factors may affect the readout of the assay.

Definition of Key Terms

NSB (Non-Specific Binding): non-immunological binding of the HRP conjugate to the well. Even in the absence of analyte a very small amount of HRP conjugate still binds to the well; the NSB is a measure of this low binding.

Principle of This Assay

This immunometric assay is based on a double-antibody "sandwich" technique. Each well of the microwell plate supplied with the kit has been coated with a recombinant mouse monoclonal antibody specific for MPO.

This antibody will bind any MPO introduced into the well. A second monoclonal antibody, conjugated to horseradish peroxidase (HRP), that recognizes DNA in the NET fragment, is added to the well forming a "sandwich." The "sandwich" is immobilized on the plate and the excess reagents are washed away. The detection of NET fragments is accomplished by measuring the enzymatic activity of HRP using the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB). After a sufficient period, the reaction is stopped with acid, forming a product with a distinct yellow color that can be measured at 450 nm. The intensity of the color is directly proportional to the amount of bound antibody-HRP conjugate, which is indicative of the presence of the NET fragments.

$$\text{Absorbance} \propto [\text{Anti-DNA HRP}] \propto [\text{NET fragment}]$$

A schematic of this process is shown in Figure 1, on page 9

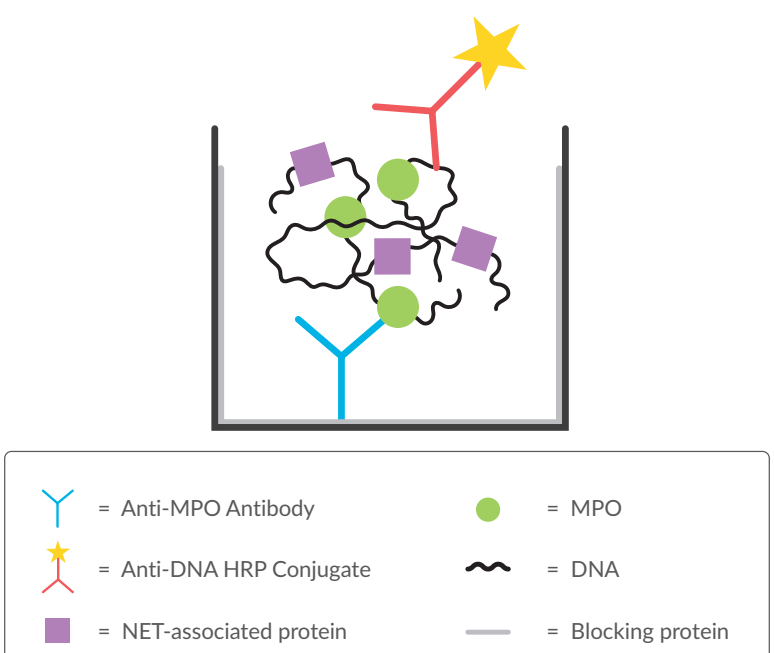

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Buffer Preparation

NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with ultrapure water. Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.

1. **Assay Buffer Preparation**

   Dilute the contents of one vial of Immunoassay Buffer F Concentrate (5X) (Item No. 401146) with 40 ml of ultrapure water and add 62.5 µl of Polysorbate 20 (Item No. 400035). Be certain to rinse the vial to remove any salts that may have precipitated. The Assay Buffer will be stable for two weeks when stored at 4°C.

2. **Wash Buffer (1X) Preparation**

   Dilute the contents of one vial of Wash Buffer Concentrate (400X) (Item No. 400062) with ultrapure water to a total volume of 2 L and add 1 ml of Polysorbate 20. The Wash Buffer (1X) will be stable for two months when stored at 4°C.

Sample Preparation

This assay has been validated in human plasma (using EDTA or citrate as an anticoagulant) and cell culture supernatant. Human serum and plasma (heparin) interfere in the assay and should not be used. Plasma must be diluted at least 1:10 with Assay Buffer prior to use in the assay. Validation data for Cayman's NET Fragment Assay Kit (MPO-DNA) (Item No. 501330) can be found on the Cayman website.

Cell culture supernatant samples may contain higher amounts of NET fragments depending on the experiment and may need to be diluted at least 1:100 with Assay Buffer prior to use in the assay.

A minimum volume of 250 µl of each diluted sample is needed to run the sample in duplicate in the assay. Dilutions should be performed using polypropylene tubes.

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

NET Fragment Control

The NET Fragment Control (Item No. 400817) is supplied as a 10X solution. Immediately before use, thaw at room temperature and dilute 25 µl into 225 µl of Assay Buffer and vortex briefly. Use the diluted control within 30 minutes. Aliquot and store unused 10X NET Fragment Control at -20°C and minimize freeze/thaw cycles.

Anti-DNA HRP Conjugate

Anti-DNA HRP Conjugate (10X) (Item No. 401143) is supplied as a concentrated stock solution of anti-DNA antibody conjugated to HRP. Prior to the assay, bring the Anti-DNA HRP Conjugate to room temperature and dilute immediately before use.

For a full plate, dilute 1.2 ml of the Anti-DNA HRP Conjugate with 10.8 ml of Assay Buffer; for a half plate, dilute 0.6 ml of the Anti-DNA HRP Conjugate with 5.4 ml of Assay Buffer. Discard any unused Anti-DNA HRP Conjugate (1X). Store Anti-DNA HRP Conjugate (10X) stock solution at -20°C and minimize freeze/thaw cycles.

Plate Set Up

The 96-well plate included with this kit is supplied ready to use. It is not necessary to rinse the plate prior to adding the reagents. *NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at -20°C. Be sure the plate packet is sealed with the desiccant inside.*

Each plate or set of strips must contain control and NSB wells run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.*

A suggested plate format is show in Figure 2, below. The user may vary the location and type of wells present as necessary for each experiment. It is suggested that the contents of each well be recorded on the template sheet provided (see page 21).

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	9	9	17	17	25	25	33	33	41	41
B	2	2	10	10	18	18	26	26	34	34	42	42
C	3	3	11	11	19	19	27	27	35	35	43	43
D	4	4	12	12	20	20	28	28	36	36	44	44
E	5	5	13	13	21	21	29	29	37	37	45	45
F	6	6	14	14	22	22	30	30	38	38	46	46
G	7	7	15	15	23	23	31	31	39	39	C	C
H	8	8	16	16	24	24	32	32	40	40	NSB	NSB

1-46 = Sample Wells
C = Control Wells
NSB = Non-specific Binding Wells

Figure 2. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Addition of NET Fragment Control and Samples and First Incubation

1. Add 100 µl of the diluted NET Fragment Control or samples into the appropriate wells on the plate. Add 100 µl of Assay Buffer to the NSB wells.
2. Cover the plate with the 96-Well Cover Sheet (Item No. 400012) and incubate for 2 hours at room temperature on an orbital shaker.

Addition of Anti-DNA HRP Conjugate and Second Incubation

1. Empty the wells and rinse five times with ~300 µl Wash Buffer (1X). After the last wash, gently tap the inverted plate on absorbent paper to remove the residual wash buffer.
2. Prepare a 1X working solution of the Anti-DNA HRP Conjugate as described in the Preparation of Assay Specific Reagents section.
3. Add 100 µl of the Anti-DNA HRP Conjugate (1X) working solution to all wells.
4. Cover the plate with the 96-Well Cover Sheet and incubate for 30 minutes at room temperature on an orbital shaker.

Development of the Plate

1. Empty the wells and rinse five times with ~300 µl Wash Buffer (1X). After the last wash, gently tap the inverted plate on absorbent paper to remove the residual wash buffer.
2. Add 100 µl of TMB Substrate Solution (Item No. 400074) to each well of the plate.
3. Cover the plate with the 96-Well Cover Sheet. Optimum development is obtained by using an orbital shaker at room temperature for 30 minutes, protected from light.
4. Remove plate cover being careful to keep TMB Substrate Solution from splashing on the cover. *NOTE: Any loss of TMB Substrate Solution will affect the absorbance readings.*
5. DO NOT WASH THE PLATE. Add 100 µl of HRP Stop Solution (Item No. 10011355) to each well of the plate. Blue wells should turn yellow and colorless wells should remain colorless. *NOTE: The stop solution in this kit contains an acid. Wear appropriate protection and use caution when handling this solution.*

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, *etc.*
2. Read the plate at a wavelength of 450 nm.

ANALYSIS

Interpretation of the Results

The user is responsible for establishing and validating criteria for interpretation of sample results appropriate for their intended application. An absorbance ratio of the provided control to the NSB greater than 4:1 demonstrates acceptable assay performance. If this criterion is not met, refer to the **Troubleshooting** table on page 18.

Digestion of DNA as Confirmation of Assay Specificity

Aliquots of human plasma (citrate and EDTA) were spiked with A23187-stimulated PMN supernatant and incubated at 37°C with 15 U/ml of S7 nuclease. At various time-points, digestion was halted by adding EDTA to a final concentration of 15 mM. Samples were then diluted 1:10 with Assay Buffer prior to analysis using Cayman's NET Fragment Assay Kit (MPO-DNA). Digestion of NET-associated DNA by S7 nuclease reduces the assay signal in a time-dependent manner and confirms specificity of the assay for intact NET fragments. NET-associated signal is retained longer in plasma collected with EDTA as the anti-coagulant, as EDTA inhibits nuclease through chelation of divalent metal ions required for enzymatic function.

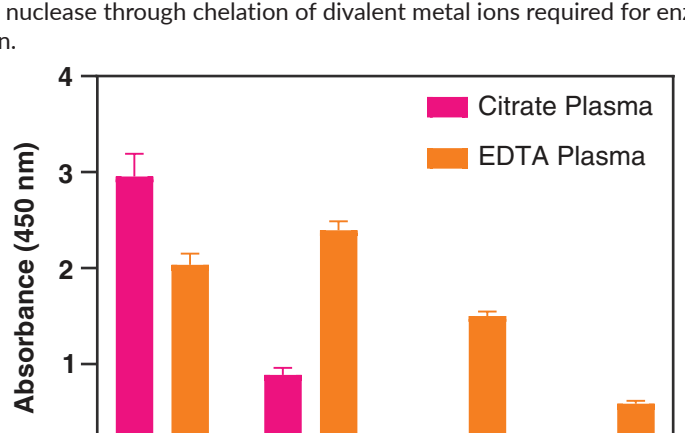

Figure 3. Loss of signal with DNA digestion

RESOURCES

Troubleshooting

Problem	Possible Causes
Erratic values; dispersion of replicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique C. NET fragments can contribute to variability due to their inherent size variation
High NSB (>0.30 O.D.)	A. TMB substrate has been contaminated B. Poor washing; ensure proper washing C. Exposure of NSB wells to control or samples D. Preparing Assay Buffer incorrectly E. Contamination of Assay Buffer
No development in control wells	A. Components may have degraded B. Dilution error in preparing reagents
High control wells (>2.5 O.D.)	A. Contaminated buffer (fungal or bacterial growth); remake Assay Buffer B. Control diluted incorrectly
No development in sample wells	A. Samples are too dilute B. No NET fragments present C. Digestion of DNA in samples or clearance of NETs

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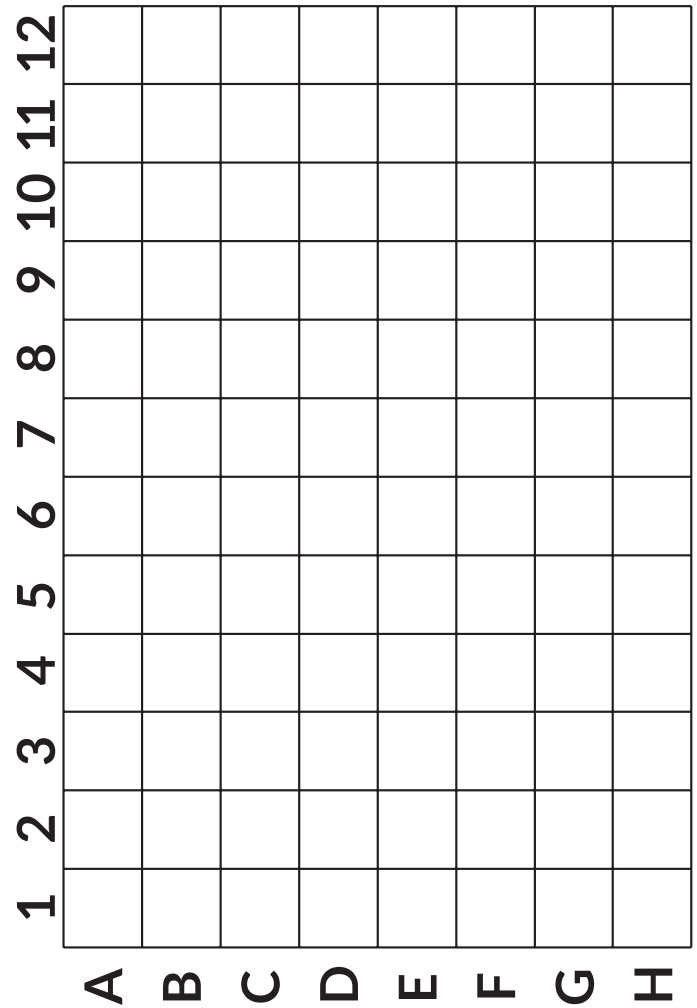
Assay Summary

Procedure	Control & Samples	NSB
Dilute and mix	Mix all reagents gently	
Control and Samples	100 µl	--
Assay Buffer	--	100 µl
First Incubation	Seal plate and incubate for 2 hours at room temperature on an orbital shaker	
Aspirate	Aspirate wells and wash 5 x ~300 µl with Wash Buffer (1X)	
Anti-DNA HRP Conjugate (1X)	100 µl	
Second Incubation	Seal plate and incubate for 30 minutes at room temperature on an orbital shaker	
Aspirate	Aspirate wells and wash 5 x ~300 µl with Wash Buffer (1X)	
Apply TMB Substrate	100 µl	
Develop	Seal plate and incubate for 30 minutes at room temperature on an orbital shaker, protected from light.	
DO NOT WASH. Add HRP Stop Solution	100 µl	
Read	Read absorbance at 450 nm	

Table 1. Assay summary



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.

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