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CAYMAN CURRENTS

Cayman CHEMICAL

NEUTROPHIL BIOLOGY

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Neutrophil Defensive NETworks

by James L. Mobley, Ph.D.

Neutrophils have been recognized for their role in the engulfment and destruction of invasive pathogenic microorganisms for more than a century. The critical role played by neutrophils in immune defense is underscored by the severity of diseases resulting from neutrophil absence or dysfunction, including Chronic Granulomatous Disease (CGD) and Leukocyte Adhesion Deficiency (LAD).[1] There are several features that make neutrophils the "special forces" of the innate immune response[2]:

1. They can be rapidly produced in very large numbers in response to a threat.
2. They are rapidly mobilized to the site of pathogen invasion.
3. They are capable of accurately identifying the enemy.
4. They carry multiple weapons capable of destroying the threat.

Production, Recruitment and Mobilization

In humans, 50-70% of white blood cells (leukocytes) in the bloodstream are neutrophils. These cells are mass-produced, fully differentiated, and rapidly replaced. They are generated in the bone marrow at a rate of 100 billion per day, and released into the circulation where they function or die within 1-2 days. They do not reproduce. In rodents, neutrophils constitute only 7-10% of circulating leukocytes, making the isolation and study of rodent peripheral blood neutrophils particularly challenging. The nature and consequence of this species-specific difference in neutrophil frequency is poorly understood.[3]

In all mammals, neutrophils are rapidly mobilized and recruited to the site of infection. The process of neutrophil recruitment has been thoroughly elucidated over the past twenty years and consists of three distinct steps. First, selectin-mediated rolling adhesion to the vascular endothelial cells slows the progression of the neutrophil in the circulation. Next, integrin-mediated firm adhesion promotes the arrest of forward motion. Finally, transendothelial migration allows the neutrophil to

▶ *continue to page 2*

leave the circulation and crawl into the underlying infected tissue. Various chemoattractants, including leukotriene B_4 (LTB_4), act as homing signals to guide the extravasated neutrophils directly to the inflammatory site.[4]

Recognition

Once the neutrophil finds its way to the site of infection, its encounter with the pathogen is mediated by one or more pattern recognition receptors (PRRs) that bind to specific pathogen-associated molecular patterns (PAMPs) that are common to multiple invasive microorganisms.[5] PRRs, in general, function in three different ways: Interacting directly with the PAMP on the cell surface, interacting directly with the PAMP in the cytoplasm, or interacting indirectly with the PAMP on the cell surface. The directly-binding cell surface PRRs recognize and respond to multiple ligands including bacterial lipopolysaccharide (*via* TLR4), peptidoglycan (TLR2), lipoteichoic acids (TLR2), bacterial DNA (TLR9), flagellin (TLR5), viral RNA (TLR3, TLR7, and TLR8), fungal β-glucan (Dectin-1) and N-formylated bacterial peptides (fMLP receptor). Intracellular PRRs include receptors that recognize bacterial peptidoglycan (NOD1) and muramyl dipeptide (NOD2). Other pattern recognition receptors are secreted from the cell (neutrophils or other immune cells) to encounter the pathogen at a distance. These include the pentraxin family members C-reactive protein and serum amyloid P, components of the complement cascade, and immunoglobulins. Once bound to their pathogen targets, these PRRs bind to the neutrophils through multiple distinct immunoglobulin Fc receptors or complement receptors. The interaction of PRRs with PAMPs promotes the phagocytosis of the pathogen, and the initiation of signal transduction cascades that trigger the activation of the arsenal of neutrophil anti-microbial weapons.

Defensive Weaponry

Neutrophils, along with eosinophils, basophils, and mast cells, are collectively called "granulocytes" because of the high number of granules contained within their cytoplasm.[6] Neutrophil primary granules, also called azurophilic granules, are thought to serve a lysosome-like function for the killing and digestion of pathogens contained within phagocytic vacuoles. Primary granules contain a plethora of antimicrobial agents including myeloperoxidase, phospholipase A_2, acid hydrolase, neutrophil elastase, lysozyme, cathepsin G, proteinase 3, iNOS, and several cationic peptides. These primary granules are rarely secreted, as their contents could be toxic to surrounding tissues. Neutrophil secondary granules, also called specific granules, contain alkaline phosphatase, NADPH oxidase, collagenase, lactoferrin, and cathelcidin (LL-37). Secondary granules are secretory in function, expelling their contents to the extracellular space. However, secondary granules also fuse with phagocytic vacuoles and primary granules, adding their contents to those of the primary granules. The NADPH oxidase contained within the secondary granules is required for the "respiratory burst," the generation of reactive oxygen species (ROS) including superoxide and hydrogen peroxide. Myeloperoxidase from the primary granules transforms hydrogen peroxide and chloride into hypochlorous acid, the active ingredient of chlorine bleach. Nitric oxide produced by iNOS reacts with superoxide to form microbicidal peroxynitrite. Once the infection has been eliminated, neutrophils undergo apoptosis, the process of programmed cell death that prevents the release of dangerous antimicrobial agents, thereby reducing damage to surrounding tissue. Apoptotic neutrophils are efficiently phagocytosed by macrophages that follow neutrophils into the inflammatory site.

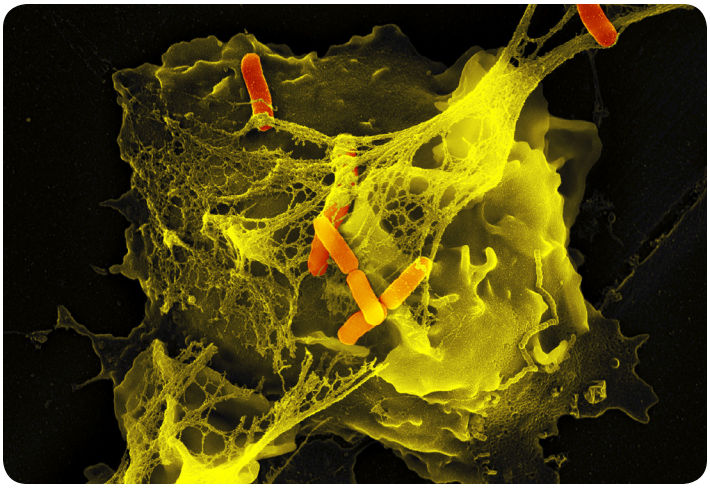

Figure 1 - Stimulated neutrophil with NETs and some trapped *Shigella* bacteria (orange). Colored scanning electron micrograph. © Max Planck Institute for Infection Biology

Neutrophil Extracellular Traps (NETs)

One important component of the neutrophil antimicrobial response remained undiscovered until 2004 when Arturo Zychlinsky's lab described the production of NETs.[7] As the name implies, NETs resemble web-like structures that the neutrophil weaves from DNA, histones, and antimicrobial macromolecules and extrudes into the extracellular space. The induction of NET formation, their composition, function, and removal are the subject of intense research.

Induction of NETs

The initial reports of NET formation *in vitro* used powerful activation stimuli (PMA, A-23187, LPS) to promote NET formation over a 3-4 hour timeframe from highly purified human peripheral blood neutrophils. NET formation was initially assessed using fluorescent DNA-staining dyes for microscopy or by detection using microplate fluorescence readers.[8] Later, DNA-independent assays were developed to measure other NET components that were released from NETs through the actions of nuclease.[9] More recently, intravital microscopy has been employed to follow NET formation in living animals as it occurs in real time and under physiological conditions. Seminal studies from Paul Kubes *et al*. have shown that in mice, NET formation is dependent upon an interaction with platelets.[10] High doses of LPS injected *in vivo* activate platelets to adhere to neutrophils and initiate NET formation. This process appears to be dependent upon platelet thromboxane A_2 (TXA_2), and can be inhibited by TXA_2 receptor antagonists.[11] This *in vivo* NET formation occurs more quickly (20 minutes) than PMA-induced NET formation *in vitro*, and does not result in the death of the neutrophil. Rather, the neutrophil body, devoid of a nucleus, is still able to move about in search of additional bacteria to engulf.

NET Formation and Composition

In the years since their discovery, the major structural components of NETs have been identified, including dsDNA, histones, myeloperoxidase (MPO), neutrophil elastase (NE), lactotransferrin, and defensin peptides.[12] Thus, it appears that NETs form as a result of the mixture of nuclear chromatin with the contents of the primary (alpha) granules. The exact mechanism of NET formation remains unclear, but several intermediate steps have been identified. Early in the process there appears to be a requirement of reactive oxygen species generated from the actions of NADPH oxidase. Neutrophils deficient in NADPH oxidase do not form NETs.[13] Next, neutrophil elastase from the primary granules migrates to the nucleus where

it degrades the linker histones (histone H1).[14] MPO also migrates to the nucleus where it enhances the process of chromatin decondensation. Finally, the nuclear enzyme peptidylarginine deiminase 4 (PAD4) is engaged.[15] PAD4 deiminates the chromatin core histones (H2A, H2B, H3, and H4), reducing their binding affinity for DNA, and thus promoting the unwinding of DNA from the core histones. At this stage the nascent NET is peppered with the antimicrobial macromolecules and extruded from the cytoplasm into the extracellular space.

NET Function and Clearance

The function of NETs was initially reported to be in the capture and killing of pathogenic microorganisms. Indeed, multiple bacterial species have been reported to die when co-cultured with NETs *in vitro*.[16] It is likely that the NET serves as a matrix for the concentration of antimicrobial agents in a confined, localized area that maximizes lethality for pathogens while minimizing damage to the surrounding host tissues. Some bacterial species are resistant to the direct killing activity of NETs; viable bacteria are released when the NETs are treated with nuclease.[17] However, the trapping of the bacteria in the NETs leads to enhanced phagocytosis by inflammatory macrophages, facilitating the indirect killing of these pathogens.

NETs contain multiple elements that if left in place too long, could induce damage to surrounding tissues or could be the targets of autoantibody formation. NETs are normally digested through the actions of serum DNase 1 followed by the removal of NET particles by scavenger receptor-mediated phagocytosis by macrophages. Inappropriate or delayed clearance of NETs or NET particles has been associated with autoantibody formation in multiple autoimmune diseases including systemic lupus erythematosus (SLE).[18] Human patients with SLE produce autoantibodies to dsDNA and histones, forming immune complexes that lead to kidney destruction. Mouse models of lupus also spontaneously produce autoantibodies against dsDNA and histones, and eventually die of kidney failure.[19] At Cayman Chemical, we hypothesized that the lupus-prone mouse strain NZBWF1 might also make autoantibodies against other NET components, and that these antibodies might be useful tools for detecting NETs by ELISA or immunohistochemistry. Indeed, we were able to produce monoclonal antibodies from NZBWF1 mice that recognize dsDNA, histones H2A, H2B, H3, and H4, myeloperoxidase, lysozyme, cathepsin G, and mCRAMP, the mouse version of LL-37 (see page 6). Many of these spontaneously generated antibodies are directed against conserved elements of their target antigens, and therefore are capable of recognizing NET components from multiple species.

PAD4

In the formation of neutrophil extracellular traps, the nuclear enzyme peptidylarginine deiminase (PAD4) modifies histones in such a way that it allows the dissociation of tightly packaged DNA from the core histone octamer. This is a critical step in NET formation. Mice deficient in PAD4 expression cannot form NETs and are susceptible to bacterial infection. Thus, the primary role of PAD4 in neutrophils appears to be in host defense, a function not recognized until 2010.[20] Prior to this report, most immunologists were already familiar with PAD4, not for its beneficial role in killing bacteria, but rather for its contribution to the pathophysiology of rheumatoid arthritis.

PAD4 catalyzes the post-translational modification of arginine to citrulline within peptides or proteins.[21] By removal of the terminal guanidine group, arginine loses a positive charge, resulting in the disruption of ionic interactions with negatively charged macromolecules, including DNA. This is thought to be the mechanism whereby PAD4 promotes chromatin decondensation. This process must be tightly regulated; unrestrained PAD4 activation leading to widespread arginine deimination could also disrupt intramolecular interactions within individual proteins, promoting protein unfolding and loss of function, and increasing susceptibility to proteolytic degradation. Furthermore, by transforming positively charged arginine to a charge neutral citrulline, PAD4 alters the ability of processed peptides containing citrulline to bind to the major histocompatibility (MHC) Class II proteins on antigen presenting cells.[22] This effect could result in the misidentification of "self" peptides as "non-self " by T lymphocytes, leading to a T cell dependent autoimmune response directed towards the citrullinated protein.

In 1994, Schellekens *et al*. first reported that patients with rheumatoid arthritis produce autoantibodies against citrulline-containing proteins.[23] This observation led to the development of highly selective assays for anti-citrullinated protein/peptide antibodies (ACAP) that are diagnostic for human rheumatoid arthritis. It also led to the identification of several proteins to which these autoantibodies are directed and include citrullinated fibrinogen, vimentin, filaggrin, and fibrin.[24] In addition, a subset of RA patients produce autoantobodies directed against the PAD4 enzyme itself.[25]

Cayman Chemical makes a variety of research tools for the study of PAD4 activity, including recombinant human PAD4, two PAD4 inhibitor screening kits, and small molecule PAD4 inhibitors Cl-amidine and F-amidine (see page 9-10). Cayman also sells PAD4-citrullinated human fibrinogen, a fluorescent chemical probe that can be used to assess the presence of citrulline within target proteins, and will soon begin to produce citrullinated vimentin and histone H3 (see page 10). Finally, Cayman produces the only commercially available assay kit for detecting anti-PAD4 autoantibodies from human plasma or serum (see page 9). As new mouse models of rheumatoid arthritis are developed that recapitulate the human disease role for PAD4, citrullinated peptides, and anti-citrullinated protein antibodies, Cayman Chemical will be there to develop the tools necessary to make research possible in this exciting new area of biology.

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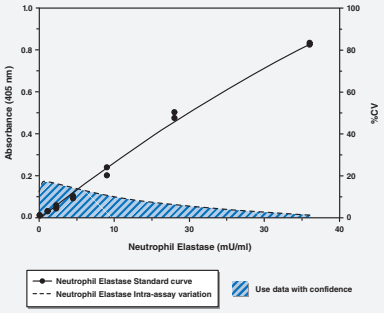
NET Quantification

Production of neutrophil extracellular traps (NETs) is a key feature of the neutrophil antimicrobial response in which neutrophils produce web-like nets from DNA, histones, and antimicrobial macromolecules upon encountering a bacterial challenge. These NETs, which are also comprised of myeloperoxidase, neutrophil elastase, and lactotransferrin, are extruded to capture and possibly kill any nearby bacteria. Once released, NETs are rapidly cleared by the action of plasma DNase and by macrophages *via* scavenger receptor-mediated phagocytosis of the NET/neutrophil complex.

NETosis Assay Kit 601010

- Induce and detect NET formation *in vitro*
- Elastase-based readout
- Non-dsDNA readout eliminates false positives from DNA
- Adaptable to multiple species



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QUESTIONS FROM THE FIELD

Can the NETosis Assay Kit (Item No. 601010) be used to detect NETs that might have already formed in whole blood, serum, or plasma?

No - the NETosis Assay Kit was designed specifically for the induction and detection of NET formation *in vitro*. It was not designed to detect NET remnants or fragments that may remain in blood, serum, or plasma after NET formation *in vivo*. To detect NET fragments, a different type of assay is required.

Can the NETosis Assay Kit (Item No. 601010) be used with mouse or rat neutrophils?

Yes - all of the reagents supplied with the kit are compatible with mouse or rat neutrophils. However, several researchers in this field have reported that it is difficult to generate NETs from mouse neutrophils obtained from bone marrow or peritoneal lavage. One published report has found that the addition of platelets to mouse neutrophils increases NET formation.[1]

Reference

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Neutrophils go Viral

Inter(NET) Video

Watch our video depicting the neutrophil's ability to defend the body against pathogenic invaders by ejecting a NET to capture and kill bacteria.

Visit the multimedia tab on the Cayman Chemical website **www.caymanchem.com/Literature/videos**

NET Components: Histones

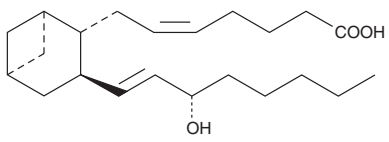
Item No.	Item Name	UniProt Accession No.
10261	Histone H2A (*Xenopus* recombinant)	Q6AZJ8
10262	Histone H2B (*Xenopus* recombinant)	Q92130
10263	Histone H3 (human recombinant)	P68431
10264	Histone H4 (human recombinant)	P62805
11010	Core Histones (human)	Native Mixture

NET-Platelet Interaction

Activated platelets have recently been identified as contributors to the induction of NET formation in several different models of the inflammatory process. The proposed mechanism involves platelet TLR4 receptor activation, which induces binding to adherent neutrophils. Sufficient platelet aggregation on neutrophils subsequently primes neutrophils to release NETs. Platelet-neutrophil interactions have demonstrated greater adhesive capacity, ROS production, and phagocytic potential. TXA_2, which elicits its effects *via* the G protein-coupled TP receptor, is a well-known activator of platelet aggregation. Agonists and antagonists of this receptor may prove useful for understanding platelet-neutrophil interactions.

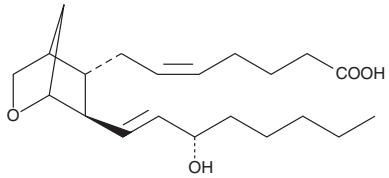
Carbocyclic Thromboxane A_2 19010

[74034-56-3] Carbocyclic TXA_2, CTA_2
MF: $C_{22}H_{36}O_3$ **FW:** 348.5 **Purity:** ≥98%
A solution in ethanol
Summary: A stable analog of TXA_2; unlike other TP receptor agonists, it inhibits platelet aggregation (IC_{50} = 4-5 µM)



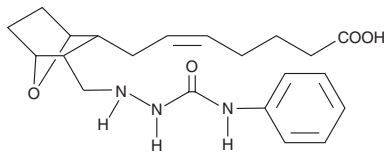
U-46619 16450

[56985-40-1] 9,11-dideoxy-9α,11α-methanoepoxy $PGF_{2α}$
MF: $C_{21}H_{34}O_4$ **FW:** 350.5 **Purity:** ≥98%
A solution in methyl acetate
Summary: TXA_2 receptor agonist; causes platelet shape change (EC_{50} = ~6 nM) and aggregation (EC_{50} = ~97 nM)



SQ 29,548 19025

[98672-91-4]
MF: $C_{21}H_{29}N_3O_4$ **FW:** 387.5 **Purity:** ≥98%
A crystalline solid
Summary: Selective TXA_2 receptor antagonist (K_i = 4.1 nM); inhibits U-46619-induced platelet aggregation (IC_{50} = 0.06 µM)



Antibodies for NET Research

Item No.	Item Name	Host	Species Reactivity	Application(s)
17088	Citrullinated Fibrinogen Monoclonal Antibody (Clone 10E9.3)	Mouse	(+) Human	ELISA, WB
17939	Histone H3 (Citrullinated R2 + R8 + R17) Monoclonal Antibody	Mouse	(+) Human	ELISA, WB
17855	Histone H3 (Citrullinated R2 + R8 + R17) Polyclonal Antibody	Rabbit	(+) Human	ELISA, WB
19822	PAD2 Monoclonal Antibody (Clone 9F7)	Mouse	(+) Human	ELISA, WB
19669	PAD4 Monoclonal Antibody (Clone 6D8)	Mouse	(+) Human	ELISA, WB
18073	Histone H1.4 (Citrullinated R53) Polyclonal Antibody	Rabbit	(+) Human	WB
15639	Myeloperoxidase Monoclonal Antibody (Clone 4E9)	Mouse	(+) Human, mouse	IF
15635	dsDNA Monoclonal Antibody (Clone 2C4)	Mouse	(+) Human, mouse	ELISA, FC, IF
15637	LL-37 Polyclonal Antibody	Rabbit	(+) Human	WB
15641	Lysozyme C Monoclonal Antibody (Clone 4C3)	Mouse	(+) Human, mouse	FC, IF
18033	Fibrinogen (α chain) Polyclonal Antibody	Rabbit	(+) Human	WB
18793	Fibrinogen (α chain) Monoclonal Antibody (Clone 6D6)	Mouse	(+) Human	WB
13535	Histone H2A Polyclonal Antibody	Rabbit	(+) Human, mouse	ELISA, WB
13784	Histone H3.3 Polyclonal Antibody	Rabbit	(+) Human, chicken, *Drosophila*, equine, mouse, opossum, ovine	IHC, WB
13543	Histone H4 Polyclonal Antibody	Rabbit	(+) Human	WB

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QUESTIONS FROM THE FIELD

Can the NETosis Assay Kit be used on non-neutrophil cell types (eosinophils, basophils, macrophages, *etc.*)?

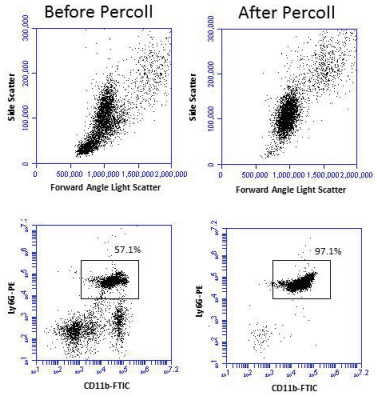
Theoretically, yes – but this assay has not been tested with cell types other than neutrophils. The buffer and stimuli (PMA, A-23187) should induce NET formation from practically any cell that is inherently capable of extracellular trap (ET) formation. Some cells simply do not have the cellular machinery to form NETs. Some cells do not produce neutrophil elastase (also known as leukocyte elastase or elastase 2), and therefore the elastase readout of the kit would not be compatible with analyzing ET formation in those cells. However, other readouts of ET formation could be applied, including analysis of soluble dsDNA release by PicoGreen. See Item No. 601010 for more information.

Neutrophil Isolation and Function

To study neutrophil defense mechanisms, including the arsenal of antimicrobial proteins and peptides they produce, it is often necessary to separate them from their *in situ* environment (generally whole blood or bone marrow). While large volumes of neutrophils can typically be isolated without difficulty from human blood samples, the isolation of neutrophils from mouse blood samples is severely limited due to an extremely low density of circulating neutrophils (neutrophils comprise only ~10% of white blood cells) and comparatively small collectable blood volumes (~1 ml/mouse). To circumvent this issue, Cayman scientists have developed a clever method to isolate neutrophils produced from a casein-induced inflammatory response in the peritoneal cavity of mice. We also designed assays useful for identifying compounds that regulate the release of defensive enzymes, such as elastase and myeloperoxidase, from isolated neutrophils and provide compounds that can manipulate neutrophil chemotaxis and aggregation.

Neutrophil (mouse) Isolation Kit 601070

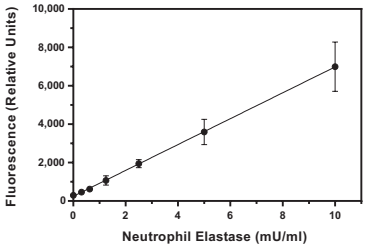
- Isolate mouse neutrophils from peritoneal lavage or bone marrow
- Circumvents issues commonplace to isolating neutrophils from mouse blood
- Includes a Percoll® density separation gradient
- Analyze purified neutrophils by flow cytometry



Flow cytometric analysis of peritoneal exudate neutrophils. Casein-elicited peritoneal cells were subjected to Percoll density separation. Prior to separation, 57.1% of the peritoneal cells were $CD11b^+Ly6G^+$ neutrophils. After Percoll, 97.1% of the cells in the pellet were neutrophils. The Percoll-purified cells also demonstrated forward angle light scatter and side scatter properties consistent with neutrophils.

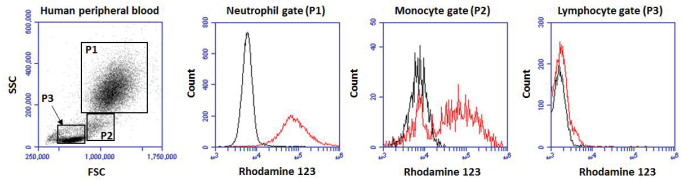
Neutrophil Elastase Activity Assay Kit 600610

- Neutrophils isolated from whole blood (protocol included)
- Measure neutrophil elastase released by neutrophils after various experimental conditions
- Employ the specific elastase substrate (Z-Ala-Ala-Ala-Ala)2Rh110
- Includes reagents to isolate neutrophils from whole blood and PMA to stimulate elastase release
- Assay performed in provided black 96-well plates
- Neutrophil (mouse) Isolation Kit available to isolate mouse neutrophils from peritoneal lavage or bone marrow



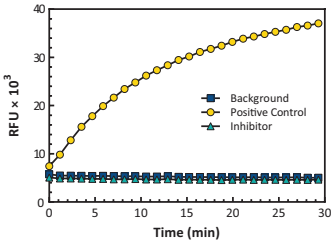
Neutrophil/Monocyte Respiratory Burst Assay Kit 601130

- Samples include whole blood from any species or cells of any type that are capable of producing a NADPH oxidase-dependent respiratory burst
- Induce and quantify a respiratory burst response
- Analyze by flow cytometry



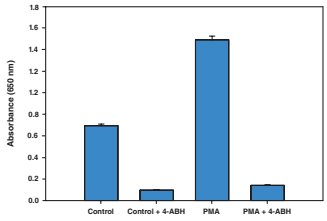
Myeloperoxidase Chlorination Fluorometric Assay Kit 10006438

- Measure MPO chlorination activity in cell lysates and purified preparations
- Assay 37 samples in duplicate
- Plate-based fluorometric measurement (ex 480-495 nm, em 515-525 nm)



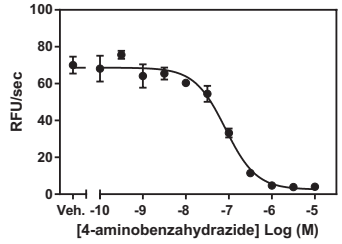
Neutrophil Myeloperoxidase Activity Assay Kit 600620

- Assay the release of enzymatically-active MPO from activated phagocytes
- Utilize TMB as a chromogenic substrate for MPO
- Includes a specific inhibitor of MPO function to verify specificity
- Includes reagents needed to isolate neutrophils from human whole blood



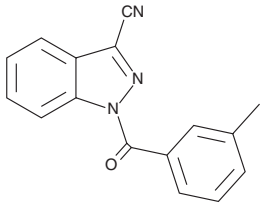
Myeloperoxidase Inhibitor Screening Assay Kit 700170

- Screen for inhibitors of MPO
- Includes reagents to measure both the chlorination and peroxidation activities
- Assay 45 samples in duplicate
- Plate-based, fluorometric measurement (ex 480-495 nm, em 515-525 nm, ex 530-540 nm, em 585-595 nm)



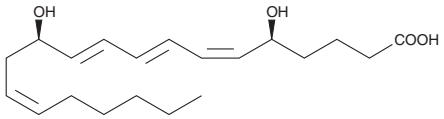
Neutrophil Elastase Inhibitor 14922

[1448314-31-5]
MF: $C_{16}H_{11}N_3O$ **FW:** 261.3 **Purity:** ≥95%
A crystalline solid
Summary: Selectively targets the binding domain of neutrophil elastase (IC_{50} = 7 nM)



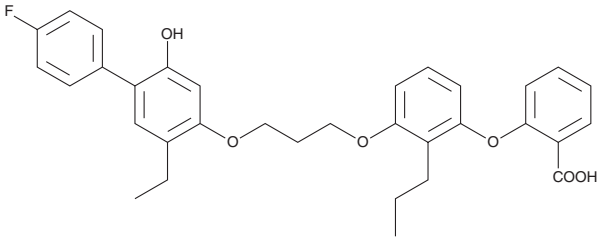
Leukotriene B_4 20110

[71160-24-2] LTB_4
MF: $C_{20}H_{32}O_4$ **FW:** 336.5 **Purity:** ≥97%
A solution in ethanol
Summary: Promotes leukocyte chemotaxis and chemokinesis (at low concentrations ~0.39 nM) as well as neutrophil aggregation/degranulation and superoxide anion production (at higher concentrations ~100 nM)



LY293111 10009768

[161172-51-6] Etalocib, VML 295
MF: $C_{33}H_{33}FO_6$ **FW:** 543.6 **Purity:** ≥98%
A solution in methyl acetate
Summary: LTB_4 receptor antagonist (IC_{50} = 17.6 nM for BLT_1) that can inhibit human neutrophil chemotaxis

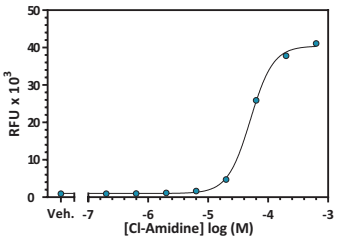


PAD Reagents

Peptidylarginine deiminases (PADs) are a family of five enzymes that catalyze the conversion of arginine to citrulline in peptides and proteins.[1] PAD4 and PAD2 are the most well-known members of this family, due to their potential roles in inflammation, epigenetics, and the pathophysiology of autoimmune diseases. PAD2 is the most widely expressed member and also the most conserved across mammalian species, implying it is the ancestral homologue of the PADs.[2] PAD2 may play a role in transcriptional regulation, as it has been shown capable of citrullinating histones, particularly H3 during mammalian reproductive cycles, when it is transcriptionally activated in the nucleus.[3] PAD2 also citrullinates vimentin in the process of macrophage apoptosis. PAD4 is located in the nucleus of neutrophils, monocytes, and other hematopoietic cells. Its known substrates include multiple histones, fibrinogen, and α-enolase.[2] PAD4-dependent citrullination of proteins is implicated in the pathophysiology of rheumatoid arthritis, due to production of anti-citrulline protein antibodies (ACPAs) and the presence of HLA Class II molecules possessing a specific epitope motif that can bind citrullinated peptides.[1,4]

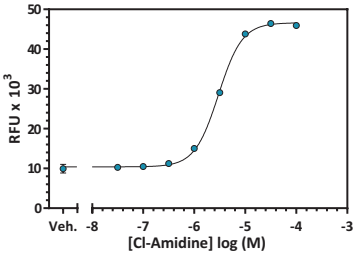
PAD2 Inhibitor Screening Assay Kit (AMC) 701390

- Screen for inhibitors of PAD2
- Assay 45 samples in duplicate
- Plate-based, fluorometric measurement (ex 355-365 nm, em 445-455 nm)
- Low background, high sensitivity



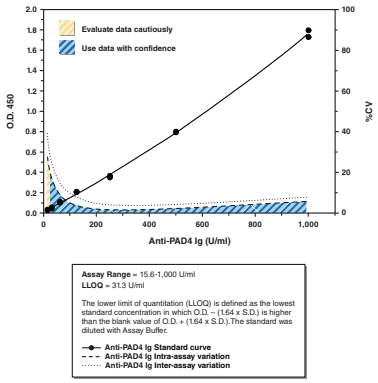
PAD4 Inhibitor Screening Assay Kit (AMC) 701320

- Screen for inhibitors of PAD4
- Assay 45 samples in duplicate
- Plate-based, fluorometric measurement (ex 355-365 nm, em 445-455 nm)
- Low background, high sensitivity
- Orthogonal assay to Cayman's PAD4 Inhibitor Screening Assay Kit (Ammonia) (Item No. 700560)



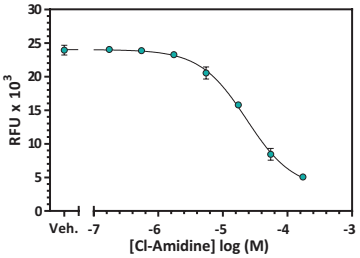
PAD4 Autoantibody ELISA Kit 500930

- Measure anti-PAD4 autoantibodies of any isotype (IgM, IgG, IgA)
- For use in human plasma and serum (no need to purify samples)
- **Assay range:** 15.6-1,000 U/ml with an LLOQ of 31.3 U/ml



PAD4 Inhibitor Screening Assay Kit (Ammonia) 700560

- Screen for inhibitors of PAD4
- Assay 45 samples in duplicate
- Plate-based, fluorometric measurement (ex 405-415 nm, em 470-480 nm)
- Orthogonal assay to Cayman's PAD4 Inhibitor Screening Assay Kit (AMC) (Item No. 701320)



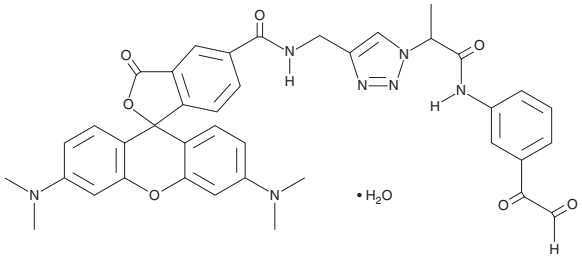
References

1. Spengler, J., Lugonja, B., Ytterberg, A.J., *et al.* Release of active peptidyl arginine deiminases by neutrophils can explain production of extracellular citrullinated autoantigens in rheumatoid arthritis synovial fluid. *Arthritis Rheumatol.* **67(12)**, 3135-3145 (2015).
2. Vossenaar, E.R., Zendman, A.J., van Venrooij, W.J., *et al.* PAD, a growing family of citrullinating enzymes: Genes, features and involvement in disease. *Bioessays* **25(11)**, 1106-1118 (2003).
3. Zhang, X., Bolt, M., Guertin, M.J., *et al.* Peptidylarginine deiminase 2-catalyzed histone H3 arginine 26 citrullination facilitates estrogen receptor a target gene activation. *Proc. Natl. Acad. Sci. USA* **109(33)**, 13331-13336 (2012).
4. Shirai, H., Blundell, T.L., and Mizuguchi, K. A novel superfamily of enzymes that catalyze the modification of guanidino groups. *Trends Biochem. Sci.* **26(8)**, 465-468 (2001).

PAD Reagents Continued

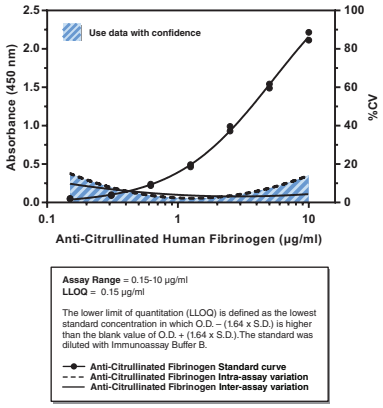
Citrulline-specific Probe 16172

- Highly sensitive
- Reacts with any citrulline-containing protein
- Analyze with fluorescent imaging (ex 532 nm, em 580 nm)



Anti-Citrullinated Human Fibrinogen Assay Kit (mouse) 501270

- Measure antibodies specific for citrullinated human fibrinogen
- Use with mouse plasma or serum
- **Assay Range:** 0.15-10 µg/ml with an LLOQ of 0.15 µg/ml
- Human fibrinogen affinity sorbent included for removal of any antibodies capable of reacting with non-citrullinated (unmodified) fibrinogen prior to analysis



PAD Inhibitors

Item No.	Item Name	Summary
17079	BB-Cl-Amidine	A potent, stable PAD4 inhibitor
10599*	Cl-Amidine (trifluoroacetate salt)	An irreversible inhibitor of PAD1, PAD3, and PAD4 (IC_{50}s = 0.8, 6.2, and 5.9 µM, respectively)
10610*	F-Amidine (trifluoroacetate salt)	An irreversible inhibitor of PAD1, PAD3, and PAD4 (IC_{50}s = 29.5, 350, and 21.6 µM, respectively)
17491	GSK121 (trifluoroacetate salt)	A parent compound for GSK484 and GSK199; inhibits citrullination of PAD4 (IC_{50} = 3.2 µM)
17489	GSK199 (hydrochloride)	A selective inhibitor of PAD4 (IC_{50} = 200 nM)
17488	GSK484 (hydrochloride)	A selective inhibitor of PAD4 (IC_{50} = 50 nM)

*Sold under license from University of South Carolina under U.S. Patent No. 7,964,363

Purified Human Fibrinogen

Item No.	Item Name	MW	Source
400076	Human Fibrinogen (PAD4 Citrullinated)	Uncitrullinated fibrinogen: α chain isoform 1 (95 kDa), α chain isoform 2 (69.8 kDa), β chain (55.9 kDa) and isoform γ-B chain (51.5 kDa)	Fibrinogen purified from human plasma citrullinated with human recombinant PAD4
18473	Human Fibrinogen (PAD2 Citrullinated)	Uncitrullinated fibrinogen: α chain isoform 1 (95 kDa), α chain isoform 2 (69.8 kDa), β chain (55.9 kDa) and isoform γ-B chain (51.5 kDa)	Fibrinogen purified from human plasma citrullinated with human recombinant PAD2

Researcher Spotlight

Where did you earn your Ph.D.? In what field?

I earned my PhD from Hannover Biomedical Research School in Germany under the mentorship of Professors Engelbert Gessner and Reinhold Schmidt. My PhD was in immunology. In my graduate study at Hannover Medical School, I examined the role of alveolar macrophage receptors in immune complex mediated lung inflammation marked by RBC and neutrophil infiltration. This basic work was later included in the Immunobiology textbook by Charles Janeway.

What can you tell us about your current research project?

Currently, I am working as an Assistant Project Scientist in the laboratory of Dr. Victor Nizet at the University of California, San Diego. Here, I am investigating the mechanisms by which we can boost the neutrophils' antimicrobial functions for the control of drug resistant pathogens. Pathogens have evolved to resist the host defense using different virulence mechanisms. For example, pathogens like *B. anthracis* inactivate MAPK pathways, *Staphylococcus aureus* inhibits complement activation, Clostridium interferes with phagocytosis, *Streptococcus* inhibits neutrophil extracellular trap release and formation of reactive oxygen species. Resilience favors pathogen survival in the host. Therefore, definitive molecular understanding of host-pathogen interactions is required to combat multiple virulent strategies. In addition, the evolution of antibiotic resistant bacteria warrants innovative strategies to make these pathogens susceptible to antibiotics.

I employ two different strategies to explore novel drug targets against bacterial pathogens. In the first strategy, I investigate the virulent mechanism of pathogens like Methicillin resistant *Staphylococcus aureus* (MRSA) and explore the possibility of perturbing it for efficient bacterial clearance by neutrophils. In a second strategy, I explore the ability of neutrophil activators (produced by host) in enhancing the bacterial clearance. The second strategy if successful can be of potential value because we can explore different pathogens that can be cleared using these activators. I am conducting these experiments in human primary neutrophils as well as in murine models. I analyze different parameters in neutrophils such as signaling pathway activation, cytokine release, reactive oxygen production, neutrophil extracellular trap formation, cell death, chemotaxis and phagocytosis. I focus on innate immune receptors and sialic acid receptors.

What are the next steps in your career? What do you plan/hope to do in the future?

I am looking forward to an academic position where I can set up my infection immunology lab. Over the years I have prepared myself for a principal investigator position that requires quality research, consistent publications, innovative ideas, and great writing skills.

Syed Raza Ali, Ph.D.
Assistant Project Scientist

University of California, San Diego
Center for Immunity, Infection & Inflammation • Nizet Lab

What piece of advice do you have for fellow postdoc researchers?

A post-doc preparing for a principal investigator position should try to acquire attributes that make him different from his peers. They should ask pertinent questions, take interest in emerging technologies, try to develop new skills, approach old problems with a new perspective and develop networks within the scientist community.

Young researchers should learn to welcome critics. Be more interactive with your colleagues- I learn more science from my colleagues than from any other place. I do not believe in competition among members of the same group. It prevents free discussion and makes the environment unhealthy for great science. And yes! Enjoy your results.

Researcher Spotlight
Want to have your research featured in the Cayman Currents? Send a brief background to **marketing@caymanchem.com**

ask Cayman
QUESTIONS FROM THE FIELD

Can the NETosis Assay Kit be used with whole blood?

No, this kit is not compatible with whole blood. Whole blood plasma contains a nuclease, DNase 1, that will destroy the NETs as they are formed. See Item No. 601010 for more information.

Helping Make Research Possible

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