Neutrophil Elastase Activity Assay Kit

Item No. 600610

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Technical Support 888.526.5351
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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
10009322	Cell-Based Assay Buffer Tablet	1 vial/3 tablets	RT
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
600613	Cell-Based Assay (Z-Ala-Ala-Ala-Ala)2Rh110	1 vial/50 µl	-20°C
601014	Human Neutrophil Elastase Assay Reagent	1 vial/50 µl	-20°C
600615	Cell-Based Assay DMF	1 vial/2.5 ml	RT
10011297	96-Well Solid Plate (black) with lid	2 plates	RT

NOTE: Histopaque® is a product of Sigma-Aldrich Co.

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: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

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

The kit should be stored at -20°C. Once it is opened, remove the Human Neutrophil Elastase Assay Reagent, Cell-Based Assay (Z-Ala-Ala-Ala-Ala)2Rh110 and PMA (1 mM) Assay Reagent from the kit and store at -20°C. Store the RBC Lysis Buffer (10X) and Cell-Based Assay Neutrophil Isolation Histopaque® at 4°C. The rest of the components may be stored at room temperature. The kit will perform as specified if used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. RPMI cell culture medium
2. Bovine Serum Albumin
3. EDTA Blood collection Tubes (can be obtained from Becton Dickinson, Product Number 366643)
4. A plate reader with the capacity to measure fluorescence using an excitation wavelength of 480-490 nm and an emission wavelength of 515-525 nm
5. Adjustable pipettes and a repeating pipettor
6. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Neutrophil elastase is the form of elastase that predominates in neutrophils, which are the most abundant type of leukocyte in human blood. The active enzyme is mainly stored within cytoplasmic azurophilic granules in the neutrophil until extruded out of the cells.[1] Upon stimulation by pathogens or pharmacological agents such as phorbol myristate acetate (PMA), neutrophil elastase is excreted from the cell and exists either as free protein or associated with networks of extracellular traps (NET).[2] Together with other proteases released from activated neutrophils, neutrophil elastase plays a critical role in degrading invading pathogens and thus provides the earliest line of defense in the immune system. In addtition to its expression in neutrophils, neutrophil elastase is also expressed in non-small cell lung cancer tumors and cell lines such as U937 cells and HL-60 cells.[3,4] Neutrophil elastase may play a critical role in tumor invasion and metastasis, due to its ability to degrade insoluble elastin and other extracellular matrix constituents. Mutations in ELA2, the gene encoding neutrophil elastase, are the major cause of the two main forms of hereditary neutropenia.[5,6] More recent studies reveal that neutrophil elastase is also involved in a variety of inflammatory human conditions such as chronic lung diseases and cancers.[7] Because of its roles in various diseases including cancers, neuthophil elastase is of interest as a potential therapeutic target.

About This Assay

Cayman's Neutrophil Elastase Activity Assay kit employs a specific non-fluorescent elastase substrate, (Z-Ala-Ala-Ala-Ala)2Rh110, which is selectively cleaved by elastase to yield the highly fluorescent compound R110. Fluorescence can be analyzed with an excitation wavelength of 485 nm and emission wavelength of 525 nm. Reagents needed to isolate neutrophils from whole blood are included in the kit, as is PMA, which is known to stimulate elastase release from neutrophils. The assay can be used to study compounds regulating elastase release in neutrophils. If a cell line known to express neutrophil elastase is used, a proper control using a specific neutrophil elastase inhibitor, such as ONO-6818 which is not provided, should be included to ensure specificity of the enzyme.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Cell-Based Assay Buffer**

   Dissolve one Cell-Based Assay Buffer Tablet (Item No. 10009322) in 100 ml of distilled water. Filter through a 0.2 µM filter before using to dilute the whole blood. Use the filtered Cell-Based Assay Buffer in a cell culture hood. This buffer should be stable for approximately one year at room temperature.

2. **RBC Lysis Buffer (10X) - (Item No. 601077)**

   On the day of use, combine 5 ml of RBC Lysis Buffer (10X) (Item No. 601077) with 45 ml distilled water. Warm to room temperature prior to use. Discard the combined reagent after 48 hours. The remaining unused RBC Lysis Buffer (10X) can be stored at 4°C for one year.

3. **Cell-Based Assay Neutrophil Isolation Histopaque® - (Item No. 600612)**

   The vial contains 25 ml of Histopaque®. It is ready to use for isolation of neutrophils from whole blood. Use the Cell-Based Assay Neutrophil Isolation Histopaque® in a cell culture hood.

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

   The vial contains 50 µl of 1 mM PMA in DMSO. To use it to stimulate neutrophil elastase release, dilute this PMA 1:10,000 in the culture medium used for your cells.

5. **(Z-Ala-Ala-Ala-Ala)2Rh110 Substrate Solution**

   Add 25 µl of the Cell-Based Assay (Z-Ala-Ala-Ala-Ala)2Rh110 (Item No.600613) to 1 ml Cell-Based Assay DMF (Item No. 600615). Prepare the Substrate Solution just before doing the assay. If you are not using all of the Cell-Based Assay (Z-Ala-Ala-Ala-Ala)2Rh110 at one time, we recommend that you store the remaining substrate at -20°C. ***Caution: DMF should be used only with solvent resistant lab ware.***

6. **Human Neutrophil Elastase Assay Reagent**

   This vial contains 50 µl human neutrophil elastase at 18 U/ml. To use the enzyme as a Positive Control, add 2 µl of the Human Neutrophil Elastase Assay Reagent (Item No. 601014) to 2 ml of the diluted Assay Buffer. Mix well. Add 100 µl of this diluted enzyme into corresponding wells in the assay plate.

   To use the enzyme to run a standard curve, obtain eight clean test tubes and label them #1 through #8. Add 9 ml of the diluted Assay Buffer into tubes #1 and 1 ml into #2-8. Transfer 5 µl of the Human Neutrophil Elastase Assay Reagent (Item No. 601014) into tube #1 and mix thoroughly. The concentration of this standard, the first point on the standard curve, is 10 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.

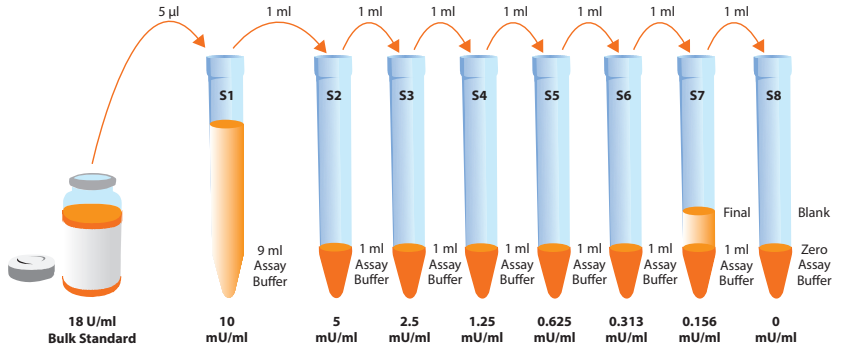

Figure 1. Preparation of the standard curve

Sample Preparation

Neutrophil Isolation

1. Collect 15 ml of whole blood in an EDTA blood collection tube.
2. Transfer the blood to a 50 ml conical tube. Rinse the blood collection tube with 15 ml of filtered Assay Buffer prepared, on page 8. Add the rinsed solution to the 50 ml conical tube.
3. Pipette 10 ml of Cell-Based Assay Neutrophil Isolation Histopaque® to a 50 ml conical tube. Slowly add 30 ml of the diluted blood on the top of Cell-Based Assay Neutrophil Isolation Histopaque®.
4. Centrifuge at 500 x g for 20-30 minutes at 18-26°C.
5. Carefully aspirate the yellowish and clear top layers and leave the reddish pellet containing neutrophils and red blood cells in the tube.
6. Pipette 30 ml of the Cell-Based Assay Red Blood Cell Lysis Buffer into the tube. Vortex to ensure mixing of the cells with the Lysis Buffer. Rock the tube on a rocker for 10-15 minutes to lyse the red blood cells.
7. Centrifuge at 1,200 x rpm for 10 minutes to pellet the neutrophils.
8. Carefully aspirate the reddish supernatant.
9. Add 5 ml of RPMI containing 1% BSA to the tube and mix well.
10. Centrifuge at 1,200 x rpm for five minutes to pellet the neutrophils.
11. Repeat steps 9 and 10 one more time.
12. Resuspend the cells in 20 ml RPMI containing 1% BSA. Mix well to ensure sufficient separation of the cells. The cells are now ready to be seeded and should be sufficient for two 96-well cell culture plates at a density of 1×10^5 - 5×10^5 cells/well.

Treatment of Cells

The following protocol is designed for a 96-well plate. For other sizes of plates the volume of medium/solution to apply to each well should be adjusted accordingly.

1. Seed a 96-well plate with the cells prepared above at 100 µl/well. Be sure to include two wells containing culture medium only for a background controls.
2. Treat the cells with experimental compounds or vehicle for two to four hours, or for the period of time used in your typical experimental protocol, in a cell culture hood. To use the PMA (1 mM) Assay Reagent (Item No. 400145), dilute the PMA solution 1:10,000 - 1:50,000 into the culture medium. PMA at these concentrations causes a significant activation of neutrophil elastase release.
3. At the end of treatment, centrifuge the plate at 1,200 x rpm for 10 minutes at 18-25°C.
4. The culture supernatant in each well is now ready for assay.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. We suggest that two wells contain culture medium without cells to be designated as background wells. Each sample should be assayed at least in duplicate. We suggest you record the contents of each well on the template sheet provided (see page 18).

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- 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.

Performing the Assay

Use the black plates included in the kit to perform the assay described below.

1. **Standard Wells** - add 100 µl of Standard (tubes #1-8) per well in the corresponding wells on the plate.
2. **Sample Wells** - Transfer 10 µl of culture supernatant from each well of the experimental plate to a corresponding well on the plate. Add 90 µl of the diluted Assay Buffer to each of the sample wells. The final volume for each sample well before addition of the substrate should be the same as that for the Standard Wells, which is 100 µl. Lesser amounts, for example 5 µl of culture supernatant, can be used when the concentration of elastase released from stimulated cells is too high and outside the standard curve. If a different volume of sample is used, be sure to adjust the amount of Sample Buffer and correct for the dilution in the calculations.
3. **Addition of the Elastase Substrate** - add 10 µl of the (Z-Ala-Ala-Ala-Ala)2Rh110 Substrate Solution prepared, on page 8, to each well.
4. Incubate the plate for 1.5 hours at 37°C.
5. Read the plate in a fluorometer at an excitation wavelength of 485 nm and an emission wavelength of 525 nm.

ANALYSIS

Calculations

1. Determine the average fluorescence of each standard.
2. Subtract the fluoresence value of the blank from itself and all other standards and samples. This is the corrected fluorescence.
3. Plot the corrected fluorescence values (from step 2 above) of each standard as a function of the final concentration of human neutrophil elastase. See Figure 2, on page 15, for a typical standard curve.
4. Determine the corrected fluorescence of each sample by subtracting the fluorescence value of background wells (wells containing culture medium without cells) from each sample.
5. Calculate the neutrophil elastase concentration of the samples using the corrected fluorescence of each sample and the equation below.

$$\text{Neutrophil Elastase (mU/ml)} = \left[\frac{\text{Fluorescence - (y-intercept)}}{\text{Slope}}\right] \times 10^{*}$$

*Accounts for a 10-fold dilution compared to the standards.

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.

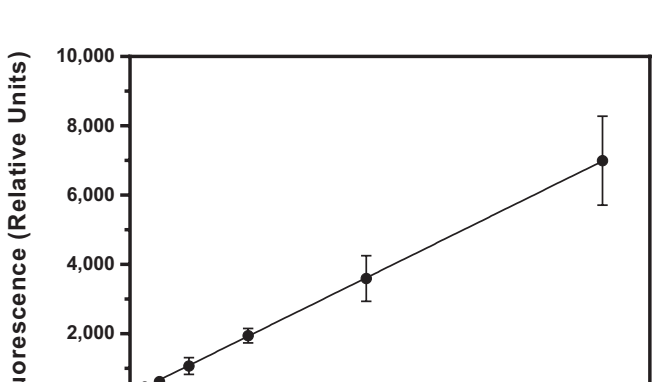

Figure 2. Human Neutrophil Elastase standard curve

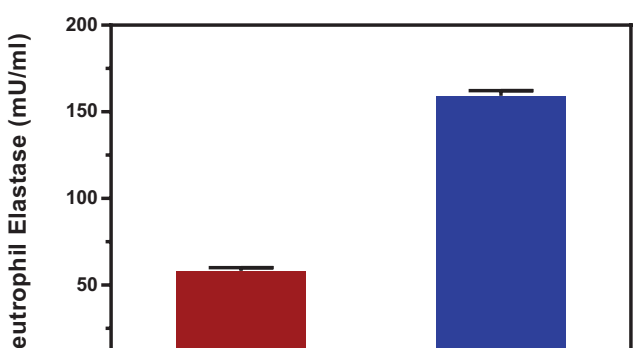

Figure 3. PMA stimulates neutrophil elastase release from human neutrophils. Human neutrophils were isolated from freshly collected human blood according to the procedure described in the booklet. The cells were then plated in RPMI containing 1% BSA in a 96-well cell culture plate. Cells were treated with either a vehicle or 50 nM PMA for three hours. The supernatant from each well (5 µl) was sampled and assayed for neutrophil elastase according to the procedure described in the booklet.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
Erratic response curve of compound treatments	Unequal number of cells in each well	Make sure each well contains the same number of cells
High level of elastase in control samples	Lysis of red blood cells is too long	Reduce the incubation time for red blood cell lysis

Reference

1. Chua, F. and Laurent, G.J. *Proc. Am. Thorac. Soc.* **3(5)**, 424-427 (2006).
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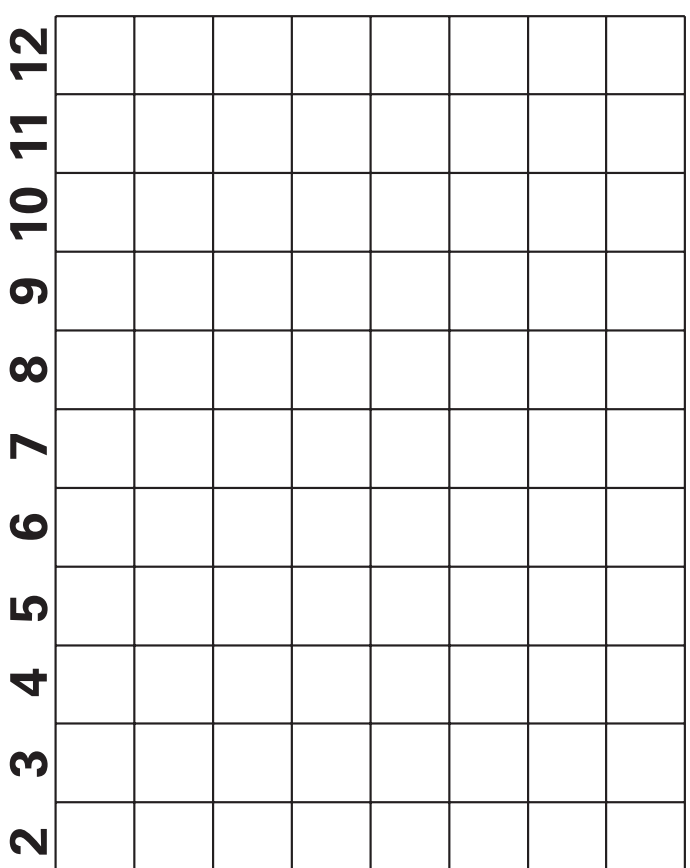
3. Yamashita, J., Ogawa, M., Abe, M., *et al. Chest* **111(4)**, 885-890 (1997)
4. Takahashi, H., Nukiwa, T., Basset, P., *et al. J. Biol. Chem.* **263(5)**, 2543-2547 (1988)
5. Horwitz, M.S., Duan, Z., Korkmaz, B., *et al. Blood* **109(5)**, 1817-1824 (2007).
6. Boxer, L.A. *Trans. Am. Clin. Climatol. Assoc.* **117**, 13-32 (2006).
7. Sato, T., Takahashi, S., Mizumoto, T., *et al. Surg. Oncol.* **15(4)**, 217-222 (2006).



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.