MILLIPORE

Mast Cell Degranulation Assay Kit

For 96 Assays

Cat. No. IMM001

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

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Introduction

Degranulation, the secretion of cytoplasmic granules, is a key step in the inflammatory response of leukocytes (e.g. basophils, neutrophils, eosinophils, and mast cells). In addition to histamine, these secretory granules contain many proinflammatory mediators including heparin, cytokines, chemokines, and many proteases (Hallgren, 2001). Tryptase, a tetrameric serine proteinase, has emerged as the major component of mast cell granules, comprising up to 20% of the total protein of mast cells derived from lung, colon and skin tissue (He, 2004; He, 1998; Schwartz, 1986). Because it is stored almost exclusively in mast cells, tryptase is a popular indicator of mast cell activation and a target for therapeutic intervention in allergic diseases. The Millipore Mast Cell Degranulation Assay Kit provides a quick, efficient and sensitive system for evaluation of tryptase activity in cell lysates, supernatants or for inhibitor screening.

Test Principle

Millipore's Mast Cell Degranulation Assay Kit provides a simple and convenient means for assaying tryptase activity. The assay is based on spectophotometric detection of the chromophore *p*-nitroaniline (*p*NA) after cleavage from the labeled substrate tosyl-gly-pro-lys-*p*NA. The free *p*NA can then be quantified using a spectrophotometer or a microtiter plate reader at 405 nm. A *p*NA Standard is provided as a comparison control.

Calcium Ionophore A23187 (Calcimycin) is included in the kit for degranulation induction of samples. Calcimycin has been a well-documented inducer of tryptase release from human mast cells (2,3).

A tryptase inhibitor, protamine, is included as a test inhibitor for screening purposes. Protamine is a potent heparin antagonist that competitively binds heparin, an essential stabilizing factor of mast cell tryptase (Hallgren, 2001; Schwartz, 1986). Protamine has been shown to inhibit both human and mouse tryptase (Hallgren, 2001). The kit's Assay Buffer contains heparin for tryptase stabilization.

Application

The Mast Cell Degranulation Assay Kit provides a quick, efficient and sensitive system for evaluation of tryptase activity in culture supernatants, cell lysates or other tryptase-containing samples. Testing of purified tryptase enzyme, *in vitro* inhibitor screening and the study of cell degranulation can also be performed with this assay.

The Millipore Mast Cell Degranulation Assay Kit is intended for research use only, and not for diagnostic or therapeutic applications.

Kit Components

1. **Tryptase Positive Control (Part No. 90572):** 20 µL of 0.1 mg/mL solution.
2. **5X Assay Buffer (Part No. 90570):** 20 mL containing 0.1% heparin.
3. **Calcium Ionophore A23187 (Part No. 90573):** 100 µL of 1 mM in DMSO.
4. **Tryptase Inhibitor (Protamine) (Part No. 90571):** 250 µL of 10 mM solution.
5. **Tryptase Substrate (tosyl-gly-pro-lys-*p*NA) (Part No. 90569):** 3.125 mg.
6. ***p*NA Standard (Part No. 90085):** 250 µL of 10 mM in DMSO.

Materials Not Supplied

- Microcentrifuge and 1.5 mL microcentrifuge tubes.
- 37°C water bath or Incubator.
- Spectrophotometer or microplate reader.
- Tissue culture treated or non-tissue culture treated plate.
- Adjustable volume pipettor with disposable tips.
- Pulse sonicator.
- 96-well microtiter plate
- 1X PBS or HBSS
- DMSO
- Ethanol

Storage

Store kit materials (as provided) at -20°C up to their expiration date. For kit use, refer to the Storage of Reagents section for component specific storage instructions.

Storage of Reagents

1. Tryptase Positive Control: Thaw vial at 2° to 8°C. Aliquot and store at -20°C up to the vial's expiration date. Avoid multiple freeze/thaw cycles.
2. 5X Assay Buffer: Dilute the 5X Assay Buffer to 100 mL with deionized water to make a 1X Assay Buffer. Stir to homogeneity and store at 2° to 8°C up to its expiration date.
3. Calcium Ionophore A23187: Thaw vial at 2° to 8°C. Aliquot and store at -20°C up to the vial's expiration date. Avoid multiple freeze/thaw cycles.
4. Tryptase Inhibitor: Thaw vial at 2° to 8°C. Aliquot and store at -20°C up to the vial's expiration date. Avoid multiple freeze/thaw cycles.
5. Tryptase Substrate: Using the 1X Assay Buffer from above, prepare a 25% ethanol stock solution and reconstitute the lyophilized Tryptase Substrate with 2 mL of this solution. Vortex until dissolved. This provides a 2.5 mM Tryptase Substrate solution. Aliquot and store at -20°C up to 3 months. Avoid multiple freeze/thaw cycles.
6. *p*NA Standard: Thaw vial at 2° to 8°C. Aliquot and store at -20°C up to the vial's expiration date. Avoid multiple freeze/thaw cycles.

   Note: *Positive Control, Substrate, pNA Standard, and Calcium Ionophore are light sensitive; amber vials or equivalent should be used. Keep materials away from light as much as possible and during storage.*

Preparation of Reagents

1. **Tryptase Positive Control Curve**
   a. Thaw Tryptase Positive Control at 2° to 8°C.
   b. Perform a dilution series (1:2 is suggested) of Tryptase Positive Control in the concentration range of 10.0 µg/mL – 0.16 µg/mL by diluting the 0.1 mg/mL stock solution in 1X Assay Buffer.

c. Transfer 10 µL of each dilution to a 96-well microtiter plate. To each well, add 170 µL of 1X Assay Buffer.

Note: The next step will initiate the reaction. If tryptase samples (see Assay Instructions) are to be tested simultaneously, the Tryptase Substrate should be added to all wells at the same time.

d. Finally, add 20 µL of reconstituted Tryptase Substrate (see Assay Instructions) to each well. Mix well and incubate 1-2 hrs at 37°C.

Note: *Assay incubation times may be extended for higher sensitivity.*

e. Read OD at 405 nm in a microplate reader.

2. **Calcium Ionophore**

The Calcium Ionophore should be pre-diluted in DMSO prior to usage (recommended **final concentration** of 1.0 - 500 nM).

Note: *For best stability, only dilute the required volume of Calcium Ionophore; retain the rest as stock solution.*

3. **Tryptase Inhibitor**

The Tryptase Inhibitor (Protamine) may be diluted in 1X Assay Buffer prior to usage (recommended **final concentration** of 1.0 - 100 µM).

Note: *For best stability, only dilute the required volume of Tryptase Inhibitor; retain the rest as stock solution.*

4. **Generating a pNA Standard Curve**

Prepare a dilution series (1:2 is suggested) of *p*NA solutions in the concentration range of 10 µM – 1 mM by diluting the provided *p*NA Standard (10 mM) in 1X Assay buffer. Add 200 µl of each dilution to a well. Include 200 µl of 1X assay buffer as a blank. Read OD at 405 nm in a microplate reader.

Preparation of Mast Cell Samples

1. Samples are isolated and prepared from areas known to contain a high percentage of mast cells. Although different isolation techniques are possible, references are provided for human umbilical cord (Greenfeder, 2003), colon (He, 2004), pulmonary (Schwartz, 1981; Caulfield, 1980), tonsil and skin mast cells (He, 1998). Preparations of human neutrophil, eosinophils, and monocytes can also be tested.

2. After isolating the cells, wash the cells with 1X Assay Buffer. Remove the solution.
3. Resuspend the cells with 1X Assay Buffer.
4. Count and adjust the cell concentration to 1.0 – 10.0 x 10^6 cells/mL with 1X Assay Buffer.
5. Add 1.0 mL of the cell suspension to a microcentrifuge tube.
6. For treatment with calcium ionophore (2003653), add 10 µL of solution (see Preparation of Reagents Section) to the cell suspension (recommended **final concentration** of 1.0 – 500 nM).
7. For treatment with tryptase inhibitor (2003652), add 10 µL of solution (see Preparation of Reagents Section) to the cell suspension (recommended **final concentration** of 1.0 – 100 µM).
8. Incubate the cells in a 37°C, 5% CO_2 incubator for 60 minutes.
9. Collection of tryptase sample:
    a. Supernatant: Centrifuge the cell suspension at 700 x *g*. Carefully collect the supernatant, leaving the cell pellet. Store at 2° to 8°C.
    b. Lysate: Centrifuge the cell suspension at 700 x *g*. Carefully aspirate the supernatant and discard. Wash cell pellet once with cold PBS. Centrifuge and discard supernatant. Resuspend the pellet in 1mL of 1X Assay Buffer. Sonicate the suspension with a pulse sonicator until cells are thoroughly lysed. Centrifuge down the cell debris, collecting the lysate sample. Store at 2° to 8°C.
10. Continue with the Assay Instructions.

Assay Instructions

Use the table below as a guide for preparation of assay samples and controls.

Sample	Assay Mixture			Tryptase Substrate	Total Volume
	Tryptase Sample	Inhibitor	1X Assay Buffer		
Buffer Blank	0 µL	0 µL	200 µL	0 µL	200 µL
Substrate Blank	0 µL	0 µL	180 µL	20 µL	200 µL
Test Sample	180 µL or X	0 µL	(180-X) µL	20 µL	200 µL
Test Sample + Inhibitor (optional)	180 µL or X	Y µL	180-(X+Y) µL	20 µL	200 µL

X = volume of cell lysate sample added if less than 180 µL.

Y = volume of inhibitor added.

1. Prepare assay mixture in a 96-well microtiter plate or standard microcentrifuge tubes, according to the above table.

   Note: If the tryptase positive control curve (see Preparation of Reagents) is to be tested simultaneously, the Tryptase Substrate should be added to all wells at the same time.

2. Initiate the colorimetric reaction by adding 20 µL of the Tryptase Substrate to each test and control well.
3. Incubate samples for 1-2 hours at 37°C.

   Note: *Assay incubation times may be extended for higher sensitivity.*

4. Read OD at 405 nm in a microplate reader.

   Note: *Background reading from cell lysates, supernatants and buffers should be subtracted from the readings before calculating fold increase in tryptase activity.*

Calculation of Results

The following charts illustrate typical results including dilutions of the *p*NA Standard and the activity curve of the Tryptase Positive Control contained in the kit. Optical Density (OD) values obtained with the Millipore Mast Cell Degranulation Assay Kit may be compared with known standards or other test samples to obtain relative activities. One should use the data below for reference only. This data should not be used to interpret actual assay results.

Figure 1: Activity Curve of Tryptase Positive Control. Tryptase Positive Control was incubated at 37°C for 30, 60 and 90 minutes with Tryptase Substrate.

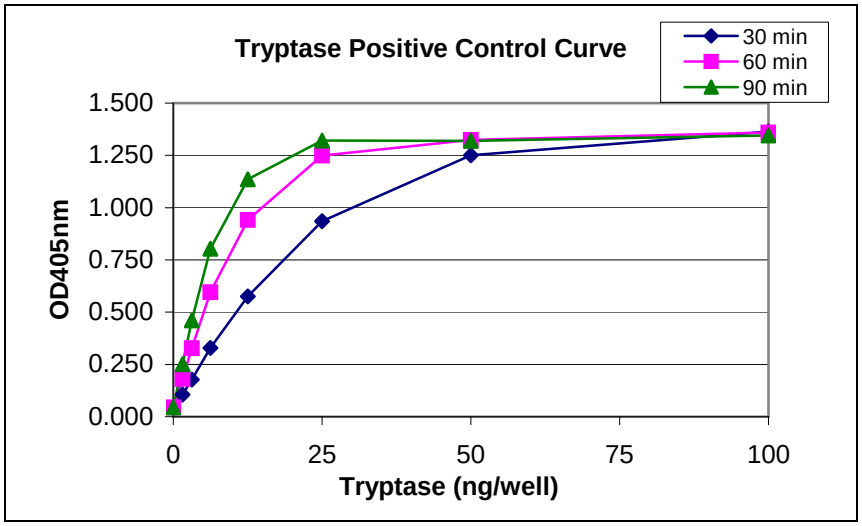


Figure 2: pNA Standard Titration Curve

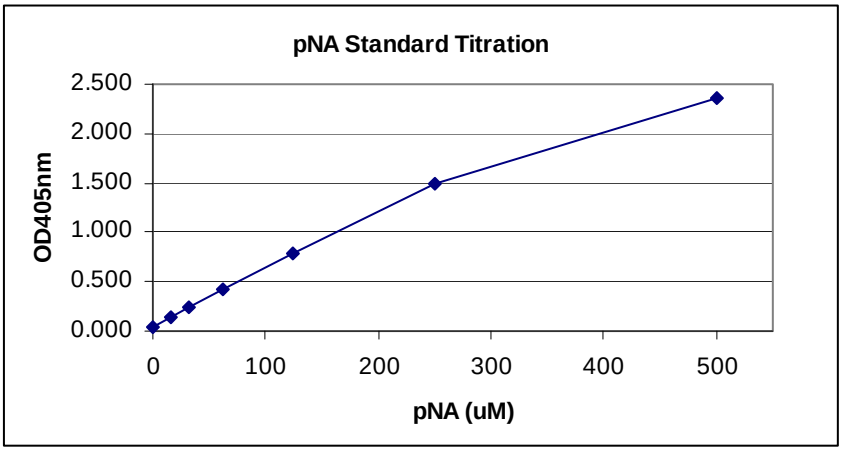


Figure 3: Inhibition of Tryptase Positive Control by Protamine. Tryptase Positive Control was pre-incubated with 100 µM Protamine for 60 minutes before adding Tryptase Substrate (2 hour incubation at 37°C).

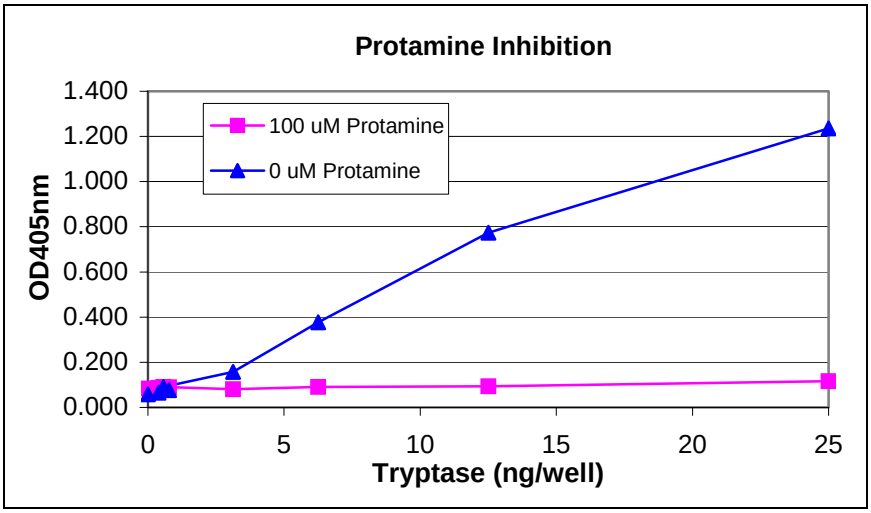
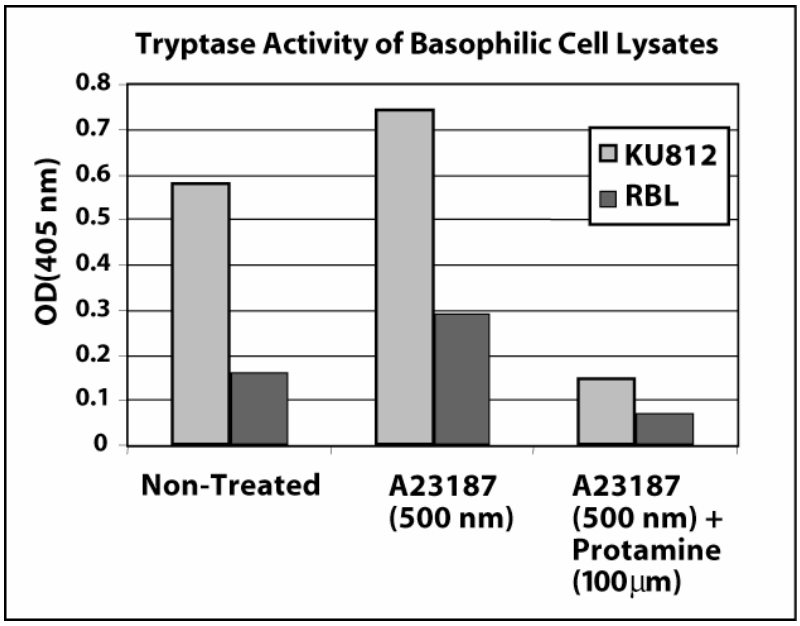


Figure 4: Tryptase Activity of Basophilic Cell Lysates. Human KU812 and Rat RBL basophils were treated with Calcium Ionophore A23187 (+/- Protamine). After the 1-hour incubation period at 37°C, the cells were lysed and tryptase activity was determined (2 hour incubation at 37°C).



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