Protease Activity Assay Kit (Red)

Catalog Number KA2524
96 assays
Version: 09

Intended for research use only
# Table of Contents

## Introduction ................................................................................................................. 3

Background ......................................................................................................................... 3

## General Information ....................................................................................................... 4

Materials Supplied .............................................................................................................. 4

Storage Instruction .............................................................................................................. 4

Materials Required but Not Supplied .................................................................................. 4

## Assay Protocol ................................................................................................................. 5

Protocol A (Measure Protease Activity in Test Samples) .................................................. 5

Protocol B (Screening Protease Inhibitors Using a Purified Enzyme) .............................. 6

## Data Analysis .................................................................................................................. 7

Calculation of Results ......................................................................................................... 7

## Resources ......................................................................................................................... 8

Appendix I .......................................................................................................................... 8

References ........................................................................................................................... 8

Plate Layout 1 ...................................................................................................................... 9

Plate Layout 2 ...................................................................................................................... 10
Introduction

Background

Protease assays are widely used for the investigation of protease inhibitors and detection of protease activities. Monitoring various protease activities has become a routine task for many biological laboratories. Some proteases have been identified as good new drug development targets.

The Protease Activity Assay Kit (Red) can be used for routine protease assays for the isolation of proteases, or for identifying the presence of contaminating proteases in samples. The kit uses a red fluorescent casein conjugate that is proven to be a generic substrate for a broad spectrum of proteases (e.g. trypsin, chymotrypsin, thermolysin, proteinase K, protease XIV, and elastase). In the intact substrate, casein is heavily labeled with a fluorescent dye, resulting in significant fluorescence quenching. Protease-catalyzed hydrolysis relieves its quenching effect, yielding brightly fluorescent dye-labeled short peptides. The increase in fluorescence intensity is directly proportional to protease activity. The assay can be performed in a convenient 96-well or 384-well microtiter plate format. Its signal can be easily read at Ex/Em = 540 /590 nm. This kit has been used for screening protease inhibitors in a HTS mode.
General Information

Materials Supplied

List of component

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component A: Protease Substrate, light sensitive.</td>
<td>300 μL</td>
</tr>
<tr>
<td>Component B: Trypsin, 5 U/μL.</td>
<td>100 μL</td>
</tr>
<tr>
<td>Component C: Assay Buffer (2X)</td>
<td>30 mL</td>
</tr>
</tbody>
</table>

Storage Instruction

Keep in freezer (-20°C) and avoid exposure to light.
All reagents are stable for 6 months after receipt when stored properly at the recommended conditions.

Materials Required but Not Supplied

- ✔ 96 microplates: Tissue culture microplate with black wall and clear bottom is recommended.
- ✔ Fluorescence microplate reader.
Assay Protocol

Protocol A (Measure Protease Activity in Test Samples)

1. Preparation of working solution

✓ Make protease substrate solution: Dilute protease substrate (Component A) at 1:100 in 2X assay buffer (Component C). Use 50 μL/well of protease substrate solution for a 96-well plate.

   Note: The 2X Assay Buffer (Component C) is designed for detecting the activity of chymotrypsin, trypsin, thermolysin, proteinase K, protease XIV, and human leukocyte elastase. For other proteases, please refer to Appendix I for the appropriate assay buffer formula.

✓ Trypsin dilution: Dilute Trypsin (5 U/μL, Component B) at 1:50 in de-ionized water to get a concentration of 0.1 U/μL.

2. Add reagents prepared in step 1 into a 96-well microplate according to table 1 and plate layout 1.

Table 1. Reagent composition for each well

<table>
<thead>
<tr>
<th>Substrate Control</th>
<th>Positive Control</th>
<th>Test Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>De-ionized water: 50 μL</td>
<td>Trypsin dilution: 50 μL</td>
<td>Protease containing samples: 50 μL</td>
</tr>
<tr>
<td>Total volume: 50 μL</td>
<td>Total volume: 50 μL</td>
<td>Total volume: 50 μL</td>
</tr>
</tbody>
</table>

Note: If less than 50 μL of protease containing biological sample is used, add ddH2O to make a total volume of 50 μL.

3. Run the enzyme reaction

✓ Add 50 μL of protease substrate solution into each well of the assay plate. Mix the reagents well.

✓ Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em = 540/590 nm.

• For kinetic reading: Immediately start measuring fluorescence intensity continuously and record data every 5 minutes for 30 minutes.

• For end-point reading: Incubate the reaction at a desired temperature for 30 to 60 minutes, protected from light. Then measure the fluorescence intensity.
Protocol B (Screening Protease Inhibitors Using a Purified Enzyme)

1. Preparation of working solution
   ✔ Prepare 1X assay buffer: Add 5 mL of de-ionized water to 5 mL of 2X Assay Buffer (Component C).
   ✔ Prepare the protease substrate solution: Dilute Protease Substrate (Component A) at 1: 20 in 1X assay buffer. Use 10 μL/well of protease substrate solution for a 96-well plate.
   Note: The 2X assay buffer (Component C) is designed for detecting the activity of chymotrypsin, trypsin, thermolysin, proteinase K, protease XIV, and human leukocyte elastase. For other proteases, please refer to Appendix I for the appropriate assay buffer formula.
   ✔ Protease dilution: Dilute the protease in 1X assay buffer to a concentration of 500-1000 nM. Each well will need 10 μL of protease diluent. Prepare an appropriate amount for all the test samples and extra for the positive control and vehicle control wells.

2. Add reagents prepared in step 1 into a 96-well microplate according to table 2 and plate layout 2.
   Table 2. Reagent composition for each well
   
<table>
<thead>
<tr>
<th>Substrate Control</th>
<th>Positive Control</th>
<th>Vehicle Control</th>
<th>Test Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X assay buffer: 90 μL</td>
<td>1X assay buffer: 80 μL</td>
<td>Vehicle*: X μL</td>
<td>Test compound: X μL</td>
</tr>
<tr>
<td>Protease dilution: 10 μL</td>
<td>Protease dilution: 10 μL</td>
<td>1X assay buffer: (80-X) μL</td>
<td>Protease dilution: 10 μL</td>
</tr>
<tr>
<td>Total volume: 90 μL</td>
<td>Total volume: 90 μL</td>
<td>Total volume: 90 μL</td>
<td>Total volume: 90 μL</td>
</tr>
</tbody>
</table>

   Note: *For each volume of test compound added into a well, the same volume of solvent used to deliver test compound needs to be checked for the effect of vehicle on the activity of protease.

3. Run the enzyme reaction
   ✔ Add 10 μL of protease substrate solution to the positive control (PC), vehicle control (VC), and test sample (TS) wells. Mix the reagents well.
   ✔ Monitor the fluorescence intensity with a fluorescence plate reader at Ex/Em = 540/590 nm.
     • For kinetic reading: Immediately start measuring fluorescence intensity continuously and record data every 5 minutes for 30 minutes.
     • For end-point reading: Incubate the reaction at a desired temperature for 30 to 60 minutes, protected from light. Then measure the fluorescence intensity.
Data Analysis

Calculation of Results

The fluorescence in the substrate control wells are used as a control, and subtracted from the values of sample wells with the enzymatic reactions.

- Plot data in the format of relative fluorescence unit (RFU) versus time for each sample (as shown in Figure 1).
- Determine the range of initial time points during which the reaction is linear. 10-15% conversion appears to be the optimal range.
- Obtain the initial reaction velocity (Vo) in RFU/min. Determine the slope of the linear portion of the data plot.
- A variety of data analyses can be run, e.g., inhibition percentage, IC50, Km, Ki, etc.

Figure 1. Trypsin protease activity was analyzed by using Protease Activity Assay Kit (Red). Protease substrate was incubated with 3 units of trypsin in the kit assay buffer. The control wells had protease substrate only (without trypsin). The fluorescence signal was measured starting from time 0 when trypsin was added using a Gemini fluorescence microplate reader (Molecular Devices ) with a filter set of Ex/Em = 540/590 nm. Samples were done in triplicate.
## Resources

### Appendix I

<table>
<thead>
<tr>
<th>Protease</th>
<th>1x Assay Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin D</td>
<td>20 mM Sodium Citrate, pH 3.0</td>
</tr>
<tr>
<td>Papain</td>
<td>20 mM sodium acetate, 20 mM cysteine, 2 mM EDTA, pH 6.5</td>
</tr>
<tr>
<td>PAE</td>
<td>20 mM sodium phosphate, pH 8.0</td>
</tr>
<tr>
<td>Pepsin</td>
<td>10 mM HCl, pH 2.0</td>
</tr>
<tr>
<td>Porcine pancreas elastase</td>
<td>10 mM Tris-HCl, pH 8.8</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>20 mM potassium phosphate buffer, pH 7.6, 150 mM NaCl</td>
</tr>
</tbody>
</table>

Note: For protocol A, 2X assay buffer is needed. For protocol B, 1x assay buffer is needed.

### References

### Plate Layout 1

<table>
<thead>
<tr>
<th></th>
<th>12</th>
<th>11</th>
<th>10</th>
<th>9</th>
<th>8</th>
<th>7</th>
<th>6</th>
<th>5</th>
<th>4</th>
<th>3</th>
<th>2</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>SC</td>
<td>PC</td>
<td>TS</td>
<td>..</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SC</td>
<td>PC</td>
<td>TS</td>
<td>..</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: SC = Substrate Control; PC = Positive Control; TS = Test Samples.
Note: SC = Substrate Control; PC = Positive Control; VC = Vehicle Control; TS = Test Samples.

Note: It is recommended to test at least three different concentrations of each test compound. All the test samples should be done in duplicates or triplicates.