SensoLyte® Rh110 Matriptase Activity Assay Kit

*Fluorimetric *

Revision number: 1.0	*Last updated: 1/27/15*
Catalog #	**AS-72241**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect Matriptase activity.
- ***Enhanced Value:*** Ample reagents to perform 100 assays in a 96-well format.
- ***High Speed:*** The entire process can be completed in one hour
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	Rh110 Matriptase substrate Ex/Em=490 nm/520 nm upon cleavage	2 mM, 50µL
Component B	Rh110 fluorescence reference standard, Ex/Em=490 nm/520 nm	2 mM,15 µL
Component C	Matriptase, human recombinant	10µg/mL, 10 µL
Component D	Assay Buffer	25 mL
Component E	Leupeptin	10mM, 15µL

Other Materials Required (but not provided)

- 96-well microplate: Black, flat bottom 96-well plate with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 520 nm with excitation at 490 nm.

Storage and Handling

- Store all kit components at -20°C, except for Component C
- Store Component C at -80°C
- Protect Components A and B from light and moisture.
- Component D can be stored at room temperature for convenience.

Introduction

Matriptase (also known as MT-SP1, ST14, TADG-15 and epithin) is a trypsin-like protease from the family of type II transmembrane serine proteases. The spectrum of known Matriptase substrates includes extracellular matrix proteins, cell adhesion molecules, ion channels, growth-factor-like proteins and other proteases. Its actions can result in protein processing, activation or degradation. Matriptase is widely expressed in virtually all epithelium and is specifically found in tumors of epithelial origin. Matriptase has been implicated in carcinogenesis including ovarian, prostate and cervical cancers and is considered a drug target for developing cancer therapeutic agent.[1, 2, 3]

The SensoLyte® Rh110 Matriptase Assay Kit provides a convenient assay for screening of enzyme inhibitors and activators or for continuous assay of enzyme activity using a fluorogenic substrate. Upon matriptase cleavage, this substrate generates the Rh110 (Rhodamine 110) fluorophore which has a bright green fluorescence and can be detected at excitation/emission=496 nm/520 nm. The longer wavelength spectra and higher extinction coefficient of Rh110 provide greater sensitivity and less interference from other reaction components. This assay can detect as low as 0.15 ng/mL active Matriptase.

Protocol

Note 1: For standard curve, please refer to Appendix II (optional).
Note 2: Please use protocol A or B based on your needs.

Protocol A. Screening Matriptase inhibitors using a purified enzyme.

1. Prepare working solutions.

Note: Bring all kit components until thawed to room temperature before starting the experiments.

1.1 Matriptase substrate solution: Dilute Matriptase substrate (Component A) 100-fold in assay buffer. Refer to Table 1. For each experiment, prepare fresh substrate solution. If not using the entire plate, adjust the amount of substrate to be diluted accordingly.

Table 1. Matriptase substrate solution for one 96-well plate (100 assays).

Components	Volume
Matriptase substrate (100X, Component A)	50 µL
Assay buffer (Component D)	4.95 mL
Total volume	5 mL

1.2 Matriptase diluent:

Dilute Matriptase enzyme (Component C) 400-fold in assay buffer (Component D). This amount of enzyme is enough for a full 96-well plate. If not using an entire plate, adjust the amount of enzyme to be diluted accordingly.

Note: Prepare enzyme diluents immediately before use. Do not vortex the enzyme solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause enzyme denaturation. Store on ice.

1.3 Inhibitor (Leupeptin): Dilute the 10 mM inhibitor solution (Component E) 1:100 in assay buffer. The diluted leupeptin solution has a concentration of 100 µM. Add 10 µl of the diluted leupeptin into each of the inhibitor control well.

2. Set up the enzymatic reaction.

2.1 Add test compounds and diluted enzyme solution to the microplate wells. The suggested volume of enzyme solution for a 96-well plate is 40 µL/well and test compound is 10 µL/ well.

2.2 Simultaneously set up the following control wells, as deemed necessary:

- Positive control contains the enzyme without test compound.
- Inhibitor control contains Matriptase enzyme and leupeptin.
- Vehicle control contains Matriptase enzyme and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains assay buffer and test compound. Some test compounds have strong autofluorescence and may give false results.
- Substrate control contains assay buffer.

2.3 Use the assay buffer to bring the total volume of all controls to 50 µL.

3. Run the enzymatic reaction.

3.1 Add 50 µL of Matriptase substrate solution into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature (37ºC usually gives higher signal-to-background ratio). Mix the reagents completely by shaking the plate gently for 30 sec.

3.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=490 nm/520 nm continuously and record data every 5 min. for 30 to 60 min.
- For end-point reading: Incubate the reaction at 37ºC for 30 to 60 min. Keep plate from direct light. Measure fluorescence intensity at Ex/Em=490 nm/520 nm.

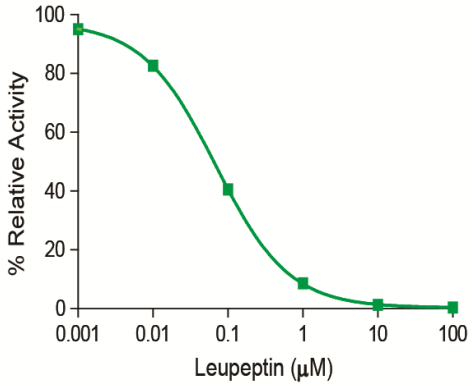

Figure 1. Inhibition of matriptase activity by leupeptin as measured with SensoLyte® Rh110 Matriptase Activity Assay Kit.

Protocol B. Measuring Matriptase activity in biological samples.

1. Prepare Matriptase containing biological samples.

1.1 Collect supernatant of cell culture media or conditioned buffer:

- Concentrate using Amicon Centrifugal Filters (Millipore).
- Use 1 to 20 µg of total protein per assay.

1.2 Prepare cell lysates:

- Wash cells with PBS. Add an appropriate amount of assay buffer (Component D). Collect the cell suspension in a microcentrifuge tube.
- Incubate the cell suspension at 4°C for 10 minutes.
- Centrifuge the cell suspension for 10 minutes at 14,000 xg, 4°C. Collect the supernatant and store at -80°C until use.

1.3 Prepare cellular membrane fractions:

- Wash cells with PBS and resuspend in ice cold PBS with protease inhibitors.
- Homogenize cells via ten aspirations through a 23-gauge needle.
- Remove intact cells and nuclei by centrifugation 5 minutes at 2,000 xg, 4°C.
- Centrifuge resulting supernatants 15 minutes at 21,000 xg, 4°C.
- Resuspend pellets with cellular membranes in assay buffer, add 0.5% NP40 to completely solubilize membrane proteins. Store samples at -80°C until use.

2. Prepare working solutions.

Note: Bring all kit components until thawed to room temperature before starting the experiments.

2.1 Matriptase substrate solution: Dilute Matriptase substrate (Component A) 1:100 in assay buffer (Component D). Refer to Table 1. For each experiment, prepare fresh substrate solution. If not using the entire plate, adjust the amount of substrate to be diluted accordingly.

Table 1. Matriptase substrate solution for one 96-well plate (100 assays)

Components	Volume
Matriptase substrate (100X, Component A)	50 µL
Assay buffer	4.95 mL
Total volume	5 mL

2.2 Matriptase diluent: If using purified Matriptase enzyme as a positive control, dilute enzyme 500-fold with assay buffer (Component D). Add 50 µl of the diluted enzyme into each of the positive control well.

Note: Prepare enzyme diluents immediately before use. Do not vortex the enzyme solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause enzyme denaturation. Store on ice.

3. Set up enzymatic reaction.

3.1 Add 50 µL of Matriptase containing biological sample.

3.2 Set up the following control wells at the same time, as deemed necessary:

- Positive control contains purified active Matriptase
- Substrate control contains assay buffer

3.3 Using the assay buffer (Component D), bring the total volume of all controls to 50 µL.

3.4 Optional: Pre-incubate the plate for 10 min. at assay temperature. Any temperature (the *assay temperature*) from room temperature to 37°C may be used, as long as the subsequent incubations are performed at the same temperature.

4. Run the enzymatic reaction.

4.1 Add 50 µL of Matriptase substrate solution into each well. For best accuracy, it is advisable to have the Matriptase substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently for 30 sec.

4.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=490 nm/520 nm continuously and record data every 5 min. for 30 to 60 min.
- For end-point reading: Incubate the reaction for 30 to 60 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

4.3 For methods of data analysis: Refer to Appendix I.

Appendix I. Data Analysis

- The fluorescence reading from the substrate control well is used as the background fluorescence. This background reading should be subtracted from the readings of the other wells containing substrate. All fluorescence readings are expressed in relative fluorescence units (RFU).
- For kinetics analysis:
  - Plot data as RFU versus time for each sample. To convert RFU to the concentration of the product of the enzymatic reaction, please refer to Appendix II for establishing a fluorescence reference standard.
  - Determine the range of initial time points during which the reaction is linear. Typically, the first 10-15% of the reaction will be the optimal range.
  - Obtain the initial reaction velocity (V_o) in RFU/min by determining the slope of the linear portion of the data plot.
  - A variety of data analyses can be done, e.g., determining inhibition %, $EC_{50,}$ IC_{50}, K_m, K_i, etc.
- For endpoint analysis:
  - Plot data as RFU versus concentration of test compounds.
  - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, etc.

Appendix II. Instrument Calibration

- Rh110 fluorescence reference standard: Dilute 2 mM Rh110 (Component B) 100-fold to 20 µM in 1X assay buffer. Do 2-fold serial dilutions to get concentrations of 10, 5, 2.5, 1.25, 0.625 and 0.312 µM, include an assay buffer blank. Add 50 µL/well of these serially diluted Rh110 reference solutions.
- Add 50 µL/well of the diluted Matriptase substrate solution (refer to Protocol A for preparation).

  Note: The Matriptase substrate solution is added to the reference standard to normalize for the intrinsic substrate fluorescence. If multiple concentrations of substrate are used, this step must be performed for each concentration.
- Measure the fluorescence of the reference standard and substrate control wells at Ex/Em=490 nm/520 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot the Rh110 reference standard curve as RFU (relative fluorescent units) versus concentration.
- The final concentrations of Rh110 reference standard are 10, 5, 2.5, 1.25, 0.625, 0.312 and 0.156 M. This reference standard is used to calibrate the variation of different instruments and different experiments. Since the proteolytic cleavage of the Rh110 substrate consists of two steps, with both the intermediate and final products having fluorescence, the Rh110 reference standard cannot serve as an indicator of the amount of Matriptase enzymatic reaction final product.

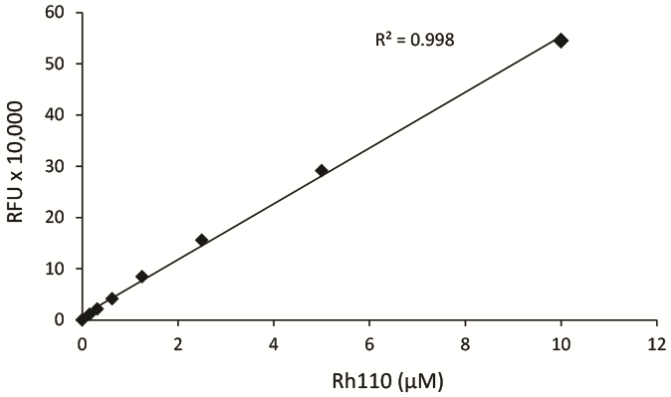

Figure 2. Rh110 reference standard. Rh110 was serially diluted in assay buffer, containing Rh110 Matriptase substrate, and fluorescence was recorded at Ex/Em=490 nm/520 nm. (Flexstation 384II, Molecular Devices)

Reference

1. Welman, A. et al. *PLOS One* **7**, e34182 (2012).
2. List, K. et al. *Mol Med* **12**, 1-3 (2006).
3. Quimbar, P. et al. *J Biol Chem* **288**, 13885 (2013).