SensoLyte® 520 Meprin α Activity Assay Kit
Fluorimetric

Revision number: 1.0	*Last updated: 02 /17/17*

Catalog #	**AS-72253**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect Meprin α 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	5-FAM /QXL™ 520 Meprin α substrate Ex/Em=490 nm/520 nm upon cleavage	0.2 mM, 50μL
Component B	5-FAM fluorescence reference standard, Ex/Em=490 nm/520 nm	0.2 mM, 15 μL
Component C	Human recombinant Meprin α	0.05 mg/mL, 20 μL
Component D	Meprin α activation buffer	100 μL
Component E	Meprin α stop solution	20 μL
Component F	2X Assay Buffer	25 mL
Component G	Inhibitor	50 μM, 200 μL

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.

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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 F can be stored at room temperature for convenience.

Introduction

Meprin α is a zinc endopeptidase of the astacin family and the metzincin superfamily. [1] Meprin α can cleave extracellular matrix proteins (pro-collagen I, fibronectin, osteopontin , SPARC), growth factors (VEGF-A, CTGF) and proteinases (MMP-1).[2-4] Evidence has suggested that Meprin α is associated with cancer proliferation, cancer migration and invasion.[2, 5-7]

The FRET substrate included in SensoLyte® 520 Meprin α Activity Assay Kit was designed to detect Meprin α activity and reduce the cross reactivity with Meprin β, ADAM10, β-secretase, BACE-2, ECEs and TACE. This assay kit can be used to detect enzyme activity in purified enzyme preparations and compound screening. Active Meprin α cleaves the FRET substrate, resulting in an increase of 5-FAM fluorescence, monitored at excitation/emission = 490 nm/520 nm. The long wavelength fluorescence of 5-FAM is also less interfered by the autofluorescence of components in biological samples and test compounds. This assay can detect as low as 0.1 ng/mL active Meprin α.

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 Meprin α 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 1X assay buffer: Add 10 ml of 2X Assay Buffer (Component F) to 10 mL deionized water. Prepare fresh 1X assay buffer for each experiment.

1.2 Meprin α substrate solution: Dilute Meprin α substrate (Component A) 100-fold in 1X assay buffer. Refer to Table 1.

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

Components	Volume
Meprin α substrate (100X, Component A)	50 µL
1X assay buffer	4.95 mL
Total volume	5 mL

1.3 Meprin α diluent:

Enzyme activation:

Add 80 µL of activation buffer (Component D) to the vial (20 µL) of Meprin α (Component C) and incubate 3 hours at 37 °C. Add 11.1 µL (1/9 of the total enzyme volume) stop solution (Component E) to the enzyme vial and mix thoroughly by pipetting. Further dilute activated

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Meprin α enzyme 40-fold in 1X assay buffer. 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: To activate specific amount of enzyme (Component C), add 4-fold volume of activation buffer (Component D) to enzyme sample and incubate for 3 hours at 37 °C. To stop the enzyme activation, add stop solution (Component E) at 1/9 of the total enzyme volume and mix thoroughly by pipetting. Further dilute activated Meprin α enzyme 40-fold in 1X assay buffer. Prepare enzyme diluents immediately before use. Do not vortex the enzyme solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store on ice.

1.4 Inhibitor (Actinonin): Dilute the 50 μM inhibitor solution (Component G) 1:20 in 1X assay buffer. The diluted Actinonin solution has a concentration of 2.5 μM. Add 10 μl of the diluted Actinonin 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 Meprin α enzyme and Actinonin.
- Vehicle control contains Meprin α enzyme and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains 1X assay buffer and test compound. Some test compounds have strong autofluorescence and may give false results.
- Substrate control contains 1X assay buffer.

2.3 Use the 1X assay buffer to bring the total volume of all controls to 50 μL.

3. Run the enzymatic reaction.

3.1 Add 50 μL of Meprin α 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 room temperature for 30 to 60 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

For methods of data analysis: Refer to Appendix I.

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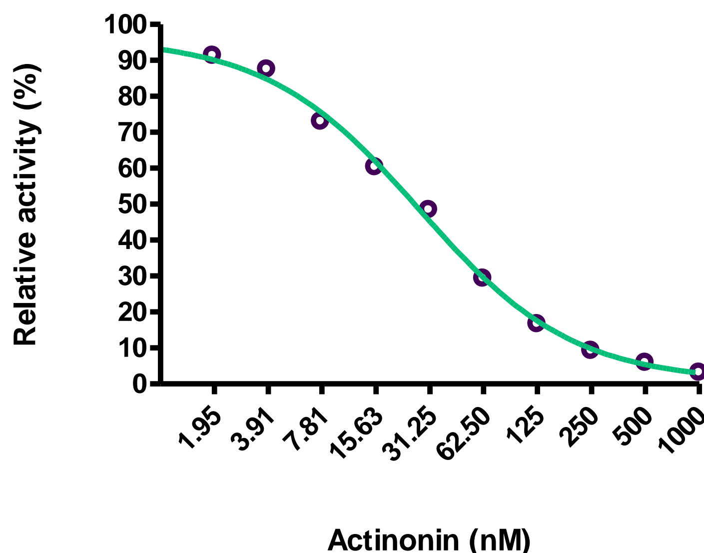

Figure 1. Inhibition of Meprin α activity by Actinonin as measured with SensoLyte® 520 Meprin α Activity Assay Kit.

Protocol B. Measuring Meprin α activity in biological samples.

Note: According to our data, the FRET substrate in this kit can also be cleaved by Neprilysin and Insulin Degrading Enzyme (IDE). The inhibitor, Actinonin, included in the assay kit specifically suppresses Meprin α activity. Therefore, it can be used with biological samples for Meprin α activity measurement.

1. Prepare Meprin α containing biological samples.

1.1 Prepare sample from cell culture medium:

- Collect medium from culture.
- Spin the medium sample for 10-15 min. at 1,000X g, 4°C.
- Collect the supernatant and store at -70°C until use.

1.2 Prepare cell lysates:

- Cells are collected by centrifugation at 500 X g for 10 min.
- Add an appropriate amount of cold 1X assay buffer to cell pellet. Collect the cell suspension to a microcentrifuge tube.
- Incubate the cell suspension on ice for at least 30 min.
- Pipette the cell suspension up and down for 5 times.
- Centrifuge the cell suspension for 5 min. at 10,000 X g, 4°C. Collect the supernatant and store at -70°C until use.

1.3 Prepare cellular membrane fractions:

- Wash cells with PBS and resuspend them in ice cold PBS with protease inhibitors.
- Samples are homogenized in cold 1X assay buffer.

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- Centrifuge homogenized cells 15 minutes at 20,000X g, 4°C.
- Wash pelleted membranes with PBS and resuspend after centrifugation in cold 1X assay buffer. Store at -70°C until use.

1.4 Prepare tissue homogenate and lysate:

- Homogenize tissue samples in cold 1X assay buffer as homogenate.
- Incubate homogenate on ice for an additional 15 min.
- Centrifuge for 15 min. at 10,000xg, 4°C. Collect the supernatant as tissue lysate. Store homogenate and/or lysate at -70°C until use.

Note: PBS is not provided.

2. Prepare working solutions.

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

2.1 Dilute Meprin α substrate (Component A) 100-fold in 2X Assay Buffer. Refer to Table 1.

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

Components	**Volume**
Meprin α substrate (100X, Component A)	50 µL
2X Assay Buffer	4.95 mL
Total volume	5 mL

2.2 Meprin α diluent:

Enzyme activation:

If using purified Meprin α enzyme as a positive control, adjust the amount of enzyme accordingly:

Add 4-fold volume of activation buffer to the enzyme and incubate 3 hours at 37°C. Stop the activation by adding 1/9 -fold of the total enzyme volume. Dilute the activated enzyme 1:50 in 1X assay buffer.

Note: Do not vortex enzyme solutions. Prolonged storage or vigorous agitation of the diluted enzyme will cause the enzyme denaturation. Store the enzyme solution on ice.

3. Set up enzymatic reaction.

3.1 Add 5-50 µL of Meprin α containing biological sample.

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

- ➢ Positive control contains purified Meprin α enzyme.
- ➢ Substrate control contains 1X assay buffer.

3.3 Using the 1X assay buffer, bring the total volume of all controls to 50 µL.

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4. **Run the enzymatic reaction.**

4.1 Add 50 µL of Meprin α substrate solution into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature (37°C usually gives higher signal-to-background ratio). 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 at room temperature 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 RFUs 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

- 5-FAM fluorescence reference standard: Dilute 0.2 mM 5-FAM reference standard (Component B) to 2 µM in 1X assay buffer. 1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.015 µM, and include 0 µM as an assay buffer blank. Add 50 µL/well of these serially diluted 5-FAM reference solutions.
- Add 50 µL/well of the diluted Meprin α substrate solution (refer to Protocol A, step 1.1 for preparation).

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Note: The Meprin α substrate solution is added to the 5-FAM reference standard to correct for the absorptive quenching by the FRET peptide. If multiple concentrations of substrate are used, this step must be performed for each concentration.

- Measure the fluorescence intensity 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 5-FAM fluorescence reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.
- The final concentrations of 5-FAM reference standard are 1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.015 and 0 μM. This reference standard is used to calibrate the variation of different instruments and different experiments. It is also an indicator of the amount of final product of the enzymatic reaction.

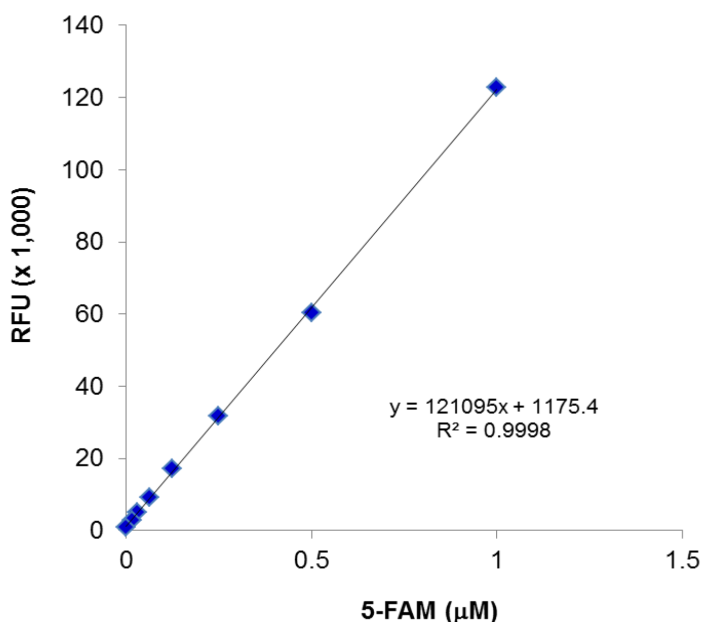

Figure 2. 5-FAM reference standard. 5-FAM was serially diluted in 1X assay buffer, containing Meprin α substrate, and the fluorescence recorded at Ex/Em=490 nm/520 nm (Flexstation 384 II, Molecular Devices).

References

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