SensoLyte® AMC Calpain Activity Assay Kit *Fluorimetric*

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72150**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect calpain activity
- ***Enhanced Value:*** It provides ample reagents to perform 100 assays in a 96-well microplate 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	Calpain Substrate, Ex/Em=354 nm/442 nm, upon cleavage	4 mM, 50 µL
Component B	AMC, Fluorescence Reference Standard, Ex/Em=354 nm/442 nm	4 mM, 10 µL
Component C	Assay Buffer	20 mL
Component D	Human Calpain	1.25 mg/mL, 40 µL
Component E	Calpain Inhibitor	50 µM, 10 µL
Component F	DTT	1M, 20 µL

Other Materials Required (but not provided)

- 96-well microplate: Black, flat-bottom, non-binding 96-well plate.
- Fluorescence microplate reader: Capable of detection at Ex/Em= 354nm /442 nm.

Storage and Handling

- Store all kit components, except Component D, at -20°C.
- Store Component D at –80°C. Aliquot as needed to avoid multiple freeze-thaw cycles.
- Protect Components A and B from light and moisture.
- Component C can be stored at room temperature for convenience.

Introduction

The calpains are a family of intracellular cysteine proteases, which consists of at least 15 ubiquitous and tissue-specific isoforms.[1] The best-characterized calpains are the isoforms μ- and m-calpain, which are also known as calpain 1 and calpain 2, respectively. Calpains are calcium-dependent and function by modulating the biological activities of many proteins through selective cleavage.[2] Studies have demonstrated that calpains are implicated in a variety of calcium-regulated cellular processes such as signal transduction, cell proliferation, differentiation, cell cycle progression, apoptosis, and platelet activation. Deregulation of calpains activities has been implicated in various pathological phenomena such as atherosclerosis, Alzheimer's disease, diabetes, and cancer.[3, 4] Calpains represent potential therapeutic targets for drug development.[5, 6]

The SensoLyte® AMC Calpain Assay Kit is optimized for detecting calpain activity. This kit contains a fluorogenic peptide substrate, Suc-LLVY-AMC. Upon calpain protease cleavage, Suc-LLVY-AMC generates the AMC fluorophore emitting bright blue fluorescence and can be monitored at excitation/emission=354/442 nm. Increase in AMC fluorescence is proportional to calpain activity. The assay can detect both calpain 1 (μ) and 2 (m) activities.

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 calpain inhibitors using purified enzyme.

1. Prepare working solutions.

Note: Allow all kit components to thaw before starting the experiment. Component D should be kept on ice after thawing.

1.1 Prepare assay buffer: Prepare fresh assay buffer for each experiment according to Table 1. **Using this DTT-containing assay buffer in all the following steps.**

Table 1. Assay buffer for one 96-well plate (100 assays)

Components	Volume
Assay buffer (Component C)	9.990 mL
1 M DTT (Component F)	10 μL
Total volume	10 mL

1.2 Calpain substrate solution: Dilute the calpain substrate (Component A) 1:100 in assay buffer according to Table 2. Prepare fresh substrate solution for each experiment.

Table 2. Calpain substrate solution for one 96-well plate (100 assays)

Components	Volume
Calpain substrate (Component A)	50 μL
Assay buffer	4.95 mL
Total volume	5 mL

1.3 Calpain enzyme diluents: Dilute the calpain enzyme (Component D) 1:100 in assay buffer according to Table 3. This amount of enzyme is enough for a full 96-well plate. If not using the 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 denaturation. Store the enzyme solution on ice.

Table 3. Calpain enzyme solution for one 96-well plate (100 assays)

Components	Volume
Calpain (Component A)	40 µL
Assay buffer	3.96 mL
Total volume	4 mL

1.4 Calpain inhibitor B27-WT: Dilute an aliquot of the 50 µM (Component E) 1:100 in assay buffer. Add 10 µL of the 0.5 µM calpain inhibitor solution to each of the inhibitor control wells in a 96-well plate.

2. Set up the enzymatic reaction.

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

2.2 Establish the following control wells at the same time, as deemed necessary:

- Positive control contains calpain enzyme without test compound.
- Inhibitor control contains calpain enzyme and inhibitor.
- Vehicle control contains calpain and vehicle used in delivering test compound (e.g. DMSO, the concentration should not 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 Using the assay buffer, bring the total volume of all controls to 50 µL.

2.4 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.

3. Run the enzymatic reaction.

3.1 Add 50 µL of calpain substrate solution from Step 1.2 into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature. 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=354 nm/442 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. Mix the reagents and measure fluorescence intensity at Ex/Em=354 nm/442 nm.

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

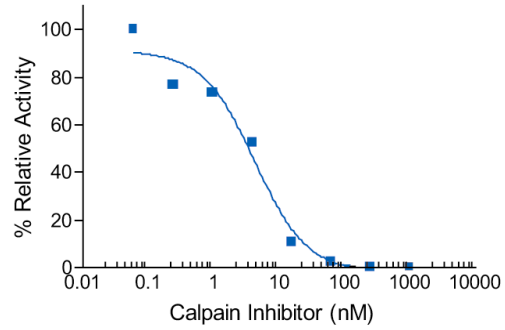

Figure 1. Inhibition of calpain activity measured with SensoLyte® AMC Calpain Activity Assay Kit.

Protocol B. Measuring calpain activity in biological samples.

1. Prepare calpain containing biological samples.

1.1 Prepare cell lysates:

- Collect cells and wash cell pellets with phosphate buffered saline (PBS).
- Lyse cells in assay buffer (see Step 2.1) and centrifuge at 12,000 x g for 10 min, 4°C.
- Collect the supernatant and store at -80°C until use.

1.2 Prepare tissue extracts:

- Collect tissues (such as liver, muscle, brain, and lens)
- Homogenize tissue samples in assay buffer (see Step 2.1) and incubate for 10 min at 4°C.
- Centrifuge at 12,000 x g for 10 min at 4°C.
- Collect the supernatant and store at -80°C until use.

Note: PBS is not provided. Cell or tissue extract should be diluted with assay buffer and used as the enzyme source to measure calpain activity.

2. Prepare working solutions.

Note: Allow all necessary kit components to thaw before starting the experiment. If using Component D, keep on ice after thawing.

2.1 Prepare assay buffer: Prepare fresh assay buffer for each experiment according to Table 1. **Using this DTT-containing assay buffer in all the following steps.**

Table 1. Assay buffer for one 96-well plate (100 assays)

Components	Volume
Assay buffer (Component C)	9.990 mL
1 M DTT (Component F)	10 µL
Total volume	10 mL

2.2 Calpain substrate solution: Dilute the calpain substrate (Component A) 1:100 in assay buffer according to Table 2. Prepare fresh substrate solution for each experiment.

Table 2. Calpain substrate solution for one 96-well plate (100 assays)

Components	Volume
Calpain substrate (Component A)	50 µL
Assay buffer	4.95 mL
Total volume	5 mL

2.3 Calpain enzyme diluents: If purified calpain enzyme is used as a positive control, then dilute the enzyme 1:125 in assay buffer. Adjust the appropriate amount of enzyme to be diluted accordingly.

3. Set up enzymatic reaction.

3.1 Add 50 µL of calpain containing biological sample.

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

- Positive control contains 50 µl of purified calpain enzyme from Step 2.3.
- Substrate control contains 50 µl of assay buffer.

3.3 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 calpain substrate solution into each well. For best accuracy, it is advisable to have the 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=354 /442 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. Mix the reagents and measure fluorescence intensity at Ex/Em=354/442 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

- AMC fluorescence reference standard: Dilute 4 mM AMC (Component B) 1:100 to 40 µM with assay buffer. Do 2-fold serial dilutions to get concentrations of 20.0, 10.0, 5.0, 2.5, 1.25, and 0.63 µM, include an assay buffer blank. Add 50 µL/well of these serially diluted AMC reference solutions into the plate.
- Add 50 µL/well of the diluted calpain substrate solution (refer to Step 1.1 for preparation) to each well. Mix the reagents by shaking the plate gently for 3 to 5 sec.

  Note: Calpain substrate solution is added to the AMC reference standard to correct for the absorption from calpain substrate. If multiple concentrations of the substrate are used, this step must be performed for each concentration.
- Measure the fluorescence intensity of the reference standard wells at Ex/Em=354 nm/442 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot the AMC standard curves as RFU (relative fluorescent units) versus concentrations as shown in Figure 2.

  Note: The final concentrations of AMC reference standard solutions are 20.0, 10.0, 5.0, 2.5, 1.25, 0.63, 0.32, 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 enzymatic reaction final product.

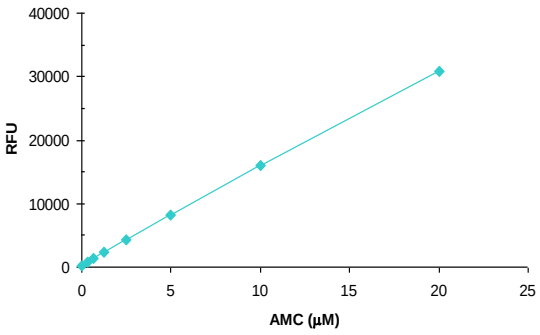

Figure 2. AMC reference standard. AMC was serially diluted in assay buffer containing substrate, and the fluorescence was recorded at Ex/Em=360/440 nm (FLX 800, Bio-Tek Instruments Inc).

References

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