SensoLyte® AMC tPA Activity Assay Kit
Fluorimetric

Revision number: 1.1 Last updated: October 2014

Catalog #	AS-72160
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect tPA 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	tPA substrate, Ex/Em=354/442 nm upon substrate cleavage	4 mM, 50 µL
Component B	AMC, fluorescence reference standard, Ex/Em=354/442 nm	4 mM, 10 µL
Component C	2X Assay Buffer	10 mL
Component D	Purified tPA enzyme	25 Units/µL, 40 µL
Component E	tPA Inhibitor	10 mM, 100 µL

Other Materials Required (but not provided)

- Microplate: Black, flat-bottom 96-well plate with non-binding surface.
- Fluorescence microplate reader: Capable of detection at Ex/Em= 354/442 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.
- Components C can be stored at room temperature for convenience.

Introduction

The primary function of tissue-type plasminogen activator (tPA) is associated with intravascular fibrinolysis through an induction of plasminogen activation. Since tPA functions as a thrombolytic enzyme in plasma, it can be used for treatment of ischemic stroke.[1] In the central nervous system, tPA promotes events associated with synaptic plasticity and cell death in a number of settings, such as cerebral ischemia and seizures. In neurons, tPA contained in vesicles can be released at the synapse after membrane depolarization or stimulation.[2, 3]

The SensoLyte® AMC tPA Assay Kit is optimized for screening of enzyme inhibitors. This kit contains a fluorogenic substrate with a high reactivity and low background. Upon protease cleavage, this substrate generates the AMC fluorophore emitting bright blue fluorescence that can be monitored at excitation/emission=354/442 nm. Increase in AMC fluorescence is proportional to tPA activity.

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 compounds using purified enzyme.

1. Prepare working solutions.

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

1.1 1X Assay buffer: Add 5 ml of 2X assay buffer (Component C) to 5 mL deionized water.

1.2 tPA substrate solution: Dilute tPA substrate (Component A) 1:100 in 1X assay buffer according to Table 1. For each experiment, prepare fresh substrate solution.

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

Components	Volume
tPA substrate (Component A)	50 µL
1X Assay buffer	4.95 mL
Total volume	5 mL

1.3 tPA diluent: Dilute tPA enzyme (Component D) 1:100 in 1X assay buffer. 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.

Table 2. tPA enzyme solution for one 96-well plate (100 assays)

Components	Volume
tPA (Component D)	40 µL
1X Assay buffer	3.96 mL
Total volume	4 mL

1.4 tPA inhibitor (Leupeptin): Add 10 µl of the 10 mM inhibitor solution (Component E) into each of the inhibitor control well of a 96-well plate.

1. 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 Simultaneously establish the following control wells, as deemed necessary:

- Positive control contains the tPA without test compound.
- Inhibitor control contains tPA and control inhibitor.
- Vehicle control contains tPA 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 Using the 1X assay buffer, bring the total volume of all controls to 50 µL.

2.4 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.

2. Run the enzymatic reaction.

3.1 Add 50 µL of tPA 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 /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.

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

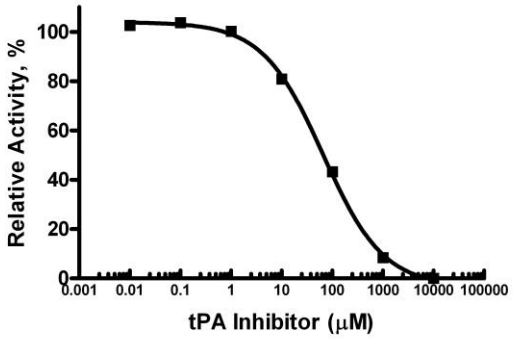

Figure 1. Inhibition of tPA activity by Leupeptin measured with SensoLyte® AMC tPA Assay Kit.

Protocol B. Measuring tPA activity in biological samples.

1. Prepare tPA containing biological samples.

1.1 Prepare blood plasma samples:

- Collect whole blood sample with anticoagulant.
- Centrifuge samples for 25 min at 2,500 x g, 4°C.
- Collect the plasma supernatant and store at -70°C until use.

1.2 Prepare cell culture supernatant samples:

- Collect cell culture media.
- Centrifuge at 3,000 x g for 15 min at 4°C to remove debris.
- Collect the supernatants and store at -70°C until use.

1.3 Prepare cell lysate samples:

- Collect cells and wash cell pellets with ice-cold phosphate buffered saline (PBS).
- Lyse cells, and centrifuge at 12,000 x g for 5 min at 4°C.
- Collect the supernatant and store at -70°C until use.

Note: PBS is not provided. Plasma, cell culture supernatant, and cell lysate should be diluted with saline and can be used as the enzyme source to measure tPA activity.

2. Prepare working solutions.

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

2.1 tPA substrate solution: Dilute tPA substrate (Component A) 1:100 in assay buffer (Component C) according to Table 1. For each experiment, prepare fresh substrate solution.

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

Components	Volume
tPA substrate (Component A)	50 µL
2X Assay buffer (Component C)	4.95 mL
Total volume	5 mL

3. Set up enzymatic reaction.

3.1 Add 50 µL of tPA containing biological sample.

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

- Positive control contains tPA positive sample or purified active tPA.
- Substrate control contains deionized water.

3.3 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.

3. Run the enzymatic reaction.

4.1 Add 50 μL of tPA substrate solution into each well. For best accuracy, it is advisable to have the tPA 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 kinetic 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) to 40 μM (1:100) with 1X assay buffer. Do 2-fold serial dilutions to get concentrations of 20, 10, 5, 2.5, 1.25 and 0.63, include an assay buffer blank. Add 50 μL/well of these serially diluted AMC reference solutions.
- Add 50 μL/well of the diluted tPA substrate solution (refer to Protocol A, step 1.2 for preparation).

Note: tPA substrate solution is added to the AMC reference standard to correct for the absorbance reading from the fluorogenic substrate. If multiple concentrations of 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 sensitivity setting as used in the enzyme reaction.
- Plot the AMC fluorescent reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.
- The final concentrations of AMC reference standard are 20, 10, 5, 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 final product of the enzymatic reaction.

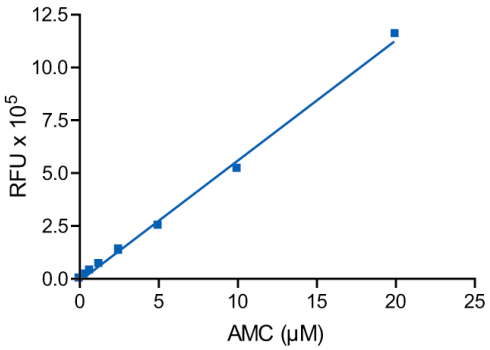

Figure 2. AMC reference standard. AMC was serially diluted in 1X assay buffer containing substrate, and the fluorescence was recorded at Ex/Em=354/442 nm. (Flexstation 384II, Molecular Devices).

References :

1. Gravanis, I. et al. *Expert Opin Ther Targets*. **12,** 159 (2008).
2. Gualandris, A. et al. *J Neurosci.* **16,** 2220 (1996).
3. Parmer, RJ. et al. *J Biol Chem.* **272,** 1976 (1997).