SensoLyte® AFC Urokinase (uPA) Activity Assay Kit *Fluorimetric*

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72159**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect uPA activity
- ***Enhanced Value:*** It provides enough 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

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Kit Components, Storage and Handling

Component	Description	Quantity
Component A	uPA Substrate, Ex/Em=380/500 nm upon enzyme cleavage	2 mM, 50 µL
Component B	AFC, Fluorescence Reference Standard, Ex/Em=380/500 nm	2 mM, 10 µL
Component C	2X Assay Buffer	10 mL
Component D	Purified Human uPA	5 Units/µL, 40 µL
Component E	uPA Inhibitor	1 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= 380/500 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

Urokinase-type plasminogen activator (uPA) is a serine protease converting the zymogen plasminogen to the active, broad-spectrum serine protease plasmin. Plasmin, in turn, mediates the pericellular proteolysis of extracellular matrix components and activates other proteases such as matrix metalloproteinases and collagenases that lead to the further degradation and remodeling of the extracellular matrix.[1,2] Besides the proteolytic function, upon binding to uPA receptor (uPAR), uPA is involved in initiating versatile intracellular signal pathways that regulate cell proliferation, adhesion, and migration through its interaction with various integrins and vitronectin.[3,4] uPA has been implicated in a number of normal and pathological processes, and recognized as a prognostic marker for various cancers as well as a target for drug discovery.[5,6]

The SensoLyte® AFC Urokinase (uPA) Assay Kit is optimized for screening of enzyme inhibitors. This kit contains a fluorogenic substrate with a high reactivity and low background. uPA cleaves the substrate resulting in release of AFC (7-amino-4-trifluoromethylcoumarin) fluorophore. Fluorescence can be monitored at Ex/Em= 380/500 nm. The substrate provided in the kit is not cleaved by tissue plasminogen activator (tPA).

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

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

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

1.3 uPA diluent: Dilute uPA 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. uPA enzyme solution for one 96-well plate (100 assays)

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

1.4 uPA inhibitor (uPA-STOP™ - synthetic uPA inhibitor): Dilute the 1 mM inhibitor solution (Component E) 1:10 to 100 µM in 1X assay buffer. Add 10 µl of the 100 µM inhibitor solution into each of the inhibitor control well of 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 Simultaneously establish the following control wells, as deemed necessary:

- Positive control contains the uPA without test compound.
- Inhibitor control contains uPA and control inhibitor.
- Vehicle control contains uPA 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.

3. Run the enzymatic reaction.

3.1 Add 50 µL of uPA 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=380 /500 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=380/500 nm.

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

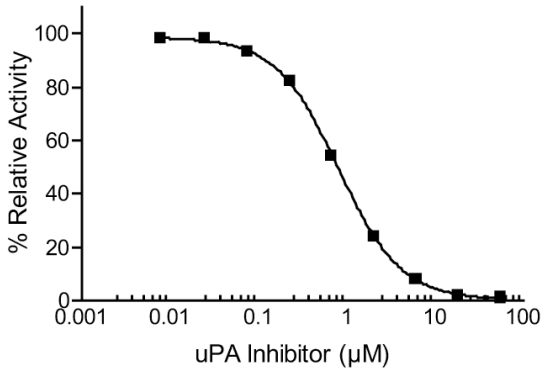

Figure 1. Inhibition of uPA activity measured with SensoLyte® AFC uPA Assay Kit.

Protocol B. Measuring uPA activity in biological samples.

1. Prepare uPA 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 uPA activity.

2. Prepare working solutions.

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

2.1 uPA substrate solution: Dilute uPA substrate (Component A) 1:100 in 2X assay buffer (Component C) according to Table 2. For each experiment, prepare fresh substrate solution.

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

Components	Volume
uPA 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 uPA containing biological sample.

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

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

3.3 Bring the total volume of all controls to 50 µL.

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 uPA substrate solution into each well. For best accuracy, it is advisable to have the uPA 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=380 /500 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=380/500 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

- AFC fluorescence reference standard: Dilute 2 mM AFC (Component B) 1:100 to 20 µM with 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 AFC reference solutions.
- Add 50 µL/well of the diluted uPA substrate solution (refer to Protocol A, step 1.2 for preparation).

  Note: uPA substrate solution is added to the AFC reference standard to correct for the absorption 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=380 nm/500 nm. Use the same sensitivity setting as used in the enzyme reaction.
- Plot the AFC fluorescent reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.
- The final concentrations of AFC reference standard are 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 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.

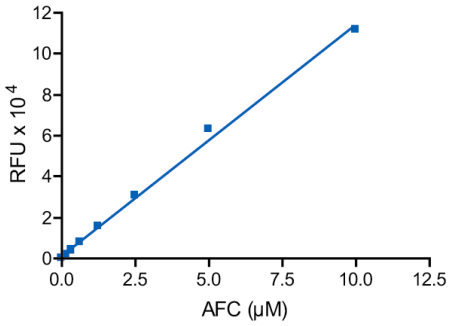

Figure 2. AFC reference standard. AFC was serially diluted in assay buffer containing substrate, and the fluorescence recorded at Ex/Em=380/500 nm. (Flexstation 384II, Molecular Devices).

References :

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