SensoLyte® 520 TACE (α-Secretase) Activity Assay Kit *Fluorimetric*

Revision number: 1.2	*Last updated: Ocober2014*
Catalog #	**AS-72085**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect TACE 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.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	QXL™ 520/5-FAM, TACE substrate, Ex/Em=490 nm/520 nm	1 mM, 50 µL
Component B	5-FAM, fluorescence reference standard, Ex/Em=490 nm/520 nm	1 mM, 10 µL
Component C	Assay Buffer	20 mL
Component D	Inhibitor of TACE	1 mM, 10 µL
Component E	Stop Solution	10 mL

Other Materials Required (but not provided)

- TACE source: The active enzyme (Calbiochem, Cat# PF133), cell culture.
- 96-well microplate: Black, flat-bottom, non-binding 96-well plate.
- 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.
- Protect Components A and B from light and from moisture.
- Components C and E can be stored at room temperature for convenience.

Introduction

TACE (TNF-α converting enzyme), also called ADAM17 or α-secretase belongs to the ADAM (A Disintegrin and Metalloprotease) family of proteins, which are involved in myogenesis, neurogenesis, and fertilization through the ectodomain shedding of cell surface proteins.[1] TACE, the first 'sheddase' to be identified and the predominant protease responsible for the generation of soluble mature TNF-α,[2] plays a crucial role in acute and chronic inflammation. Since TNF-α is a crucial mediator in the inflammatory process, considerable efforts have been made in the research and development of anti-TNF-α agents, for the purpose of reducing the severity of inflammatory responses in disease states.[3,4] The inhibition of TACE by a pharmacological agent may represent an alternative approach to modulate the effect of TNF-α,[5] TACE is also responsible for the proteolytic cleavage of amyloid precursor protein, L-selectin, transforming growth factor-α.[1,6,7]

The SensoLyte® 520 TACE Activity Assay Kit is a homogeneous assay that can be used to detect the activity of enzyme and for screening of TACE inhibitors. It contains a QXL™520/ 5-FAM FRET substrate, derived from a sequence surrounding the cleavage site of TACE.[8] In the intact FRET peptide, the fluorescence of 5-FAM is quenched by QXL™520. Active TACE cleaves FRET substrate into two separate fragments resulting in an increase of 5-FAM fluorescence which can be monitored at excitation/emission = 490 nm/520 nm. The long wavelength fluorescence of 5-FAM is less interfered by the autofluorescence of cell components and test compounds. The assay can detect as low as 3.1 ng/mL active TACE.

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 TACE inhibitors using purified enzyme.

1. Prepare working solutions.

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

1.1 TACE substrate solution: Dilute TACE substrate (Component A) 1:100 in assay buffer (Component C). For each experiment prepare fresh substrate solution.

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

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

1.2 TACE diluent: Dilute the enzyme to an appropriate concentration in assay buffer (Component C).

1.3 TACE inhibitor (TAPI-0, Peptides International Louisville, KY): Dilute the 1 mM inhibitor solution (Component D) to 10 µM in assay buffer (Component C). Add 10 µl of the 10 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 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 enzyme without test compound.
- Inhibitor control contains TACE enzyme and TAPI-0 inhibitor.
- Vehicle control contains TACE enzyme and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains assay buffer (Component C) and test compound.
- Substrate control contains assay buffer (Component C).

2.3 Using the assay buffer (Component C), 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 TACE substrate solution into each well. For best accuracy, it is advisable to have the TACE 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=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. Optional: Add 50 µL of stop solution (Component E) to each well. Mix the reagents and measure fluorescence intensity at Ex/Em=490 nm/520 nm.

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

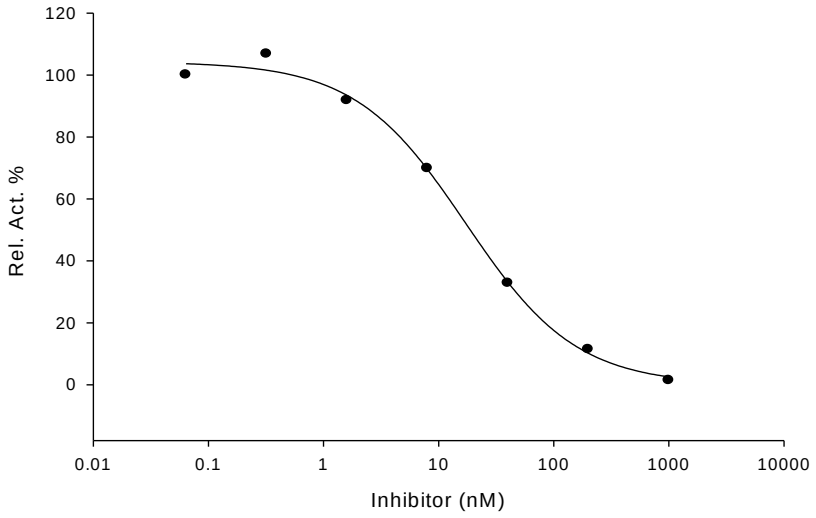

Figure 1. TAPI-0 inhibition of TACE activity measured with SensoLyte® 520 TACE Activity Assay Kit.

Protocol B. Measuring TACE activity in biological samples.

1. Prepare TACE containing biological samples.

1.1 Prepare cell lysates:

- Wash cells with PBS.
- Add an appropriate amount of assay buffer (Component C) containing 0.1% (v/v) Triton-X 100 to cells or cell pellet. 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 2,500X g, 4°C. Collect the supernatant and store at -70°C until use.

1.2 Prepare cellular membrane fractions:

- Wash cells with PBS and resuspend them in ice cold PBS with protease inhibitors.
- Lyse cells using 5 cycles of freeze-thaw
- Centrifuge lysed cells 15 minutes at 20,000X g, 4°C.
- Wash pelleted membranes with PBS and resuspend them after centrifugation in assay buffer (Component C). Store at -70°C until use.

1.3 Prepare tissue samples:

- Homogenize tissue samples in assay buffer (Component C) containing 0.1% (v/v) Triton-X 100.
- Incubate for 15 min. at 4°C.
- Centrifuge for 15 min. at 2,000xg at 4°C and collect the supernatant. Store at -70°C until use.

Note 1: Triton-X 100 and PBS are not provided.
Note 2: TACE activity also can be continuously monitored on live cells. In this case, the assay may need optimization. For example the cells could be incubated with TACE substrate (Component A) in cell medium, PBS or serum free media.

2. Prepare working solutions.

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

2.1 TACE substrate solution: Dilute TACE substrate (Component A) 1:100 in assay buffer (Component C). For each experiment prepare fresh substrate solution.

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

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

2.2 TACE diluent: If you use purified TACE as a positive control, then dilute the enzyme to an appropriate concentration in assay buffer (Component C).

3. Set up enzymatic reaction.

3.1 Add 50 µL of TACE containing biological sample.

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

- Positive control contains purified active TACE.
- Substrate control contains assay buffer.

Note: If measuring TACE activity on live cells, establish additional controls, baseline control (contains cells incubated without substrate) and medium control (contains medium only).

3.3 Using the assay buffer (Component C), 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 TACE substrate solution into each well. For best accuracy, it is advisable to have the TACE 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. Optional: Add 50 µL of stop solution (Component E) to each well. Mix the reagents and 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. If you want 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 1 mM 5-FAM (Component B) to 4 μM in assay buffer (Component C). Do 2-fold serial dilutions to get concentrations of 2, 1, 0.5, 0.25, 0.12, and 0.06 μM, include an assay buffer blank. Add 50 μL/well of these serially diluted 5-FAM reference solutions.
- Add 50 μL/well of the diluted TACE substrate solution (refer to Protocol A, step 1.1 for preparation).

  Note: The TACE substrate solution is added to the 5-FAM reference standard to correct the absorptive quenching by the FRET peptide. If multiple concentrations of substrate are used, this step must be performed for each concentration.
- Optional: If the stop solution (Component E) was added into the enzymatic reaction before taking the end-point reading, the same volume of stop solution should be added to the reference standard wells to obtain a better comparison.
- Measure the fluorescence intensity of the reference standard wells at Ex/Em=490 nm/ 520 nm. Use the same setting of sensitivity used in the enzyme reaction.
- Plot the 5-FAM fluorescent reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.
- The final concentrations of 5-FAM reference standard are 2, 1, 0.5, 0.25, 0.12, 0.06, 0.03, 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.

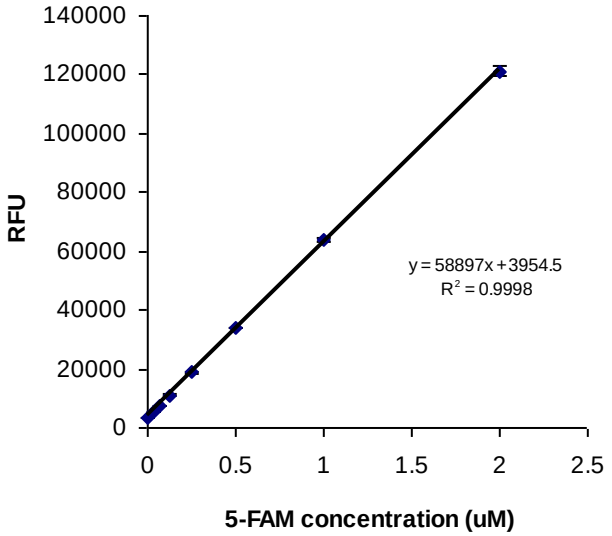

Figure 2. 5-FAM reference standard, 5-FAM was serially diluted in assay buffer containing substrate, and the fluorescence was recorded at Ex/Em=485±20 nm/ 528±20 nm. (Flexstation 384II, Molecular Devices)

References

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