SensoLyte® Homogeneous Rh110 Caspase-3/7 Assay Kit *Fluorimetric*

Revision number:1.2	*Last Updated: July 2021*
Catalog #	**AS-71141**
Kit Size	500 Assays (96-well plate)

- ***Convenient Format:*** Mix-and-read one-step homogenous assay.
- ***Optimized Performance:*** Optimal conditions for the detection of caspase-3/7 activity.
- ***Enhanced Value:*** Less expensive than the sum of individual components.
- ***High Speed:*** Minimal hands-on time.
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	Rh110 Caspase-3/7 substrate Peptide sequence=(Z-Asp-Glu-Val-Asp)$_2$-Rh110 Ex/Em=496 nm/520 nm upon caspase-3/7 cleavage	250 µL
Component B	Rh110, fluorescence reference standard Ex/Em=496 nm/520 nm	1 mM DMSO solution, 40 µL
Component C	Ac-DEVD-CHO, a known caspase-3/7 inhibitor Peptide sequence=Ac-Asp-Glu-Val-Asp-CHO	5 mM DMSO solution, 10 µL
Component D	Assay buffer	30 mL
Component E	DTT	1 M, 1.1 mL
Component F	10X Lysis Buffer	20 mL

Other Materials Required (but not provided)

- 96-well microplate: Black tissue culture microplate with or without clear bottom.
- Fluorescence microplate reader: Capable of detecting emission at 520 ±30 nm with excitation at 496±30 nm.

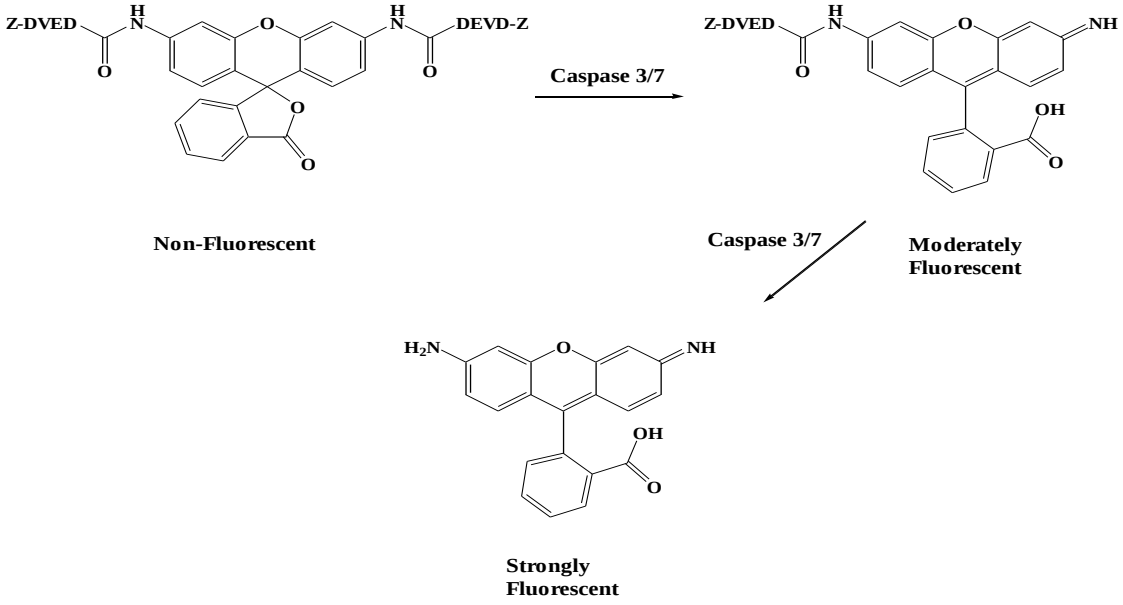
Storage and Handling

- Store all kit components at -20°C
- Protect Components A and B from light and moisture

Introduction

Apoptosis is involved in a variety of physiological and pathological events[1], ranging from normal fetal development to diseases, such as cancer[2], organ failure and neurodegenerative diseases. Central to the execution phase of apoptosis are the two closely related caspase-3 and caspase-7. They share common substrate specificity and structure, but differ completely in the sequence of their respective N-terminal regions. Both caspase-3 and caspase-7 have substrate selectivity for the amino acid sequence Asp-Glu-Val-Asp (DEVD).

The SensoLyte® Homogeneous Rh110 Caspase-3/7 Assay Kit uses $(Z\text{-DEVD})_2$-Rh110 as the fluorogenic indicator for assaying caspase-3/7 activities. Upon caspase-3/7 cleavage, $(Z\text{-DEVD})_2$-Rh110 generates the Rh110 (rhodamine 110) fluorophore that has bright green fluorescence and can be detected at excitation/emission=496 nm/520 nm (Scheme 1). The longer-wavelength spectra and higher extinction coefficient of the Rh110 provide greater sensitivity and less interference from cell components. A bi-functional assay buffer lyses the cells and provides optimal conditions for measuring enzymatic activity. Thus, this kit can measure caspase-3/7 activity in cell culture directly in a 96-well without a time-consuming cell extraction step. In cases where cells are cultured in larger plates or flasks, a lysis buffer along with a protocol for cell lysate preparation are also conveniently included in the kit. The kit can detect as low as 0.2 ng/mL active caspase-7. The kit can be used for high throughput screening of apoptosis inducers and inhibitors.



Scheme 1. Proteolytic cleavage of $(Z\text{-DEVD})_2$-Rh110.

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. Screen apoptosis inducers or inhibitors using cell culture

1. Prepare apoptotic cells.

Note: The following one-step homogeneous assay procedure is for seeding cells directly in a 96-well plate. If cells are cultured in plates larger than 96-well plates (e.g. 6-well plate or 10 cm plate), it is necessary to first prepare the cell extract. Please refer to Appendix III for details.

1.1 Seed $1X10^{3-6}$ cells per well in a 96-well microplate. Add test compounds and then incubate cells in a 37°C incubator for the desired exposure period. The suggested volume is 100 µL of cells plus 50 µL of test compounds for a total volume of 150 µL.

1.2 Set up the following controls at the same time:

- Positive control contains cells and a known apoptosis inducer.
- Negative control contains cells but no test compounds.
- Vehicle control contains cells and the vehicle used to deliver test compounds.
- Non-cell control contains growth medium but no cells.
- Test compound control contains growth medium and test compound. Some test compounds have strong autofluorescence and may give false results.

Note: Bring up the total volume of all the controls to 150 µL/well using growth medium.

2. Prepare working solutions.

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

2.1 Caspase-3/7 substrate solution: Prepare DTT-containing assay buffer by adding 40 µL of 1 M DTT (Component E) per mL of assay buffer (Component D). Dilute caspase-3/7 substrate (Component A) 1:100 in this DTT-containing assay buffer. Mix the reagents well.

Note: Prepare fresh substrate solution for each experiment.

Table 1. Caspase-3/7 substrate solution for one 96-well plate (100 assays).

Components	Volume
Caspase-3/7 substrate (Component A)	50 µL
1 M DTT (Component E)	200 µL
Assay buffer (Component D)	4.75 mL
Total volume	5 mL

3. Initiate enzymatic reaction.

3.1 Retrieve plates from the 37°C incubator if using cells cultured in a 96-well plate. Or if using cell lysate (refer to Appendix III), dispense cell extract at 150 µL/well.

3.2 Add 50 µL/well of caspase-3/7 substrate solution into each well. Mix the reagents completely by shaking on a plate shaker for 30-60 sec at 100-200 rpm. Avoid bubbles.

Note: It is not necessary to remove the culture medium from the 96-well plates. The caspase-3/7 substrate solution is a dual function solution; it lyses cells and supports optimal caspase-3/7 activity. The 10X lysis buffer (Component F) is only for preparing cell extract from plates larger than 96-well plates, for example, 6-well plate or 10 cm plate. Please refer to Appendix III for details.

3.3 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. on a plate shaker at 100-200 rpm. Keep the plate from direct light, then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

Note: If the caspase-3/7 activity is low in your samples, incubation time can be extended up to 18 hr before taking the end-point reading.

3.4 Data analysis: Refer to Appendix I.

Protocol B. Screen caspase-3/7 inducers or inhibitors using purified caspase-3/7

1. Prepare working solutions.

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

1.1 Assay buffer: Add 1 M DTT (Component E) 50-fold into assay buffer (Component D). **Use this DTT-containing assay buffer in all the following steps.**

Note: Prepare fresh DTT-containing assay buffer for each experiment.

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

Components	Volume
1 M DTT (Component E)	200 µL
Assay buffer (Component D)	9.8 mL
Total volume	10 mL

1.2 Caspase-3/7 substrate solution: Dilute caspase-3/7 substrate (Component A) 1:100 in assay buffer. Mix the reagents well.

Note: Prepare fresh substrate solution for each experiment.

Table 2. Caspase-3/7 substrate solution for one 96-well plate (100 assays).

Components	Volume
Caspase-3/7 substrate (Component A)	50 µL
Assay buffer (contains 20 mM DTT)	4.95 mL
Total volume	5 mL

- Caspase-3/7 diluent: Dilute caspase-3/7 to an appropriate concentration in assay buffer.

  Note: Prepare enzyme diluent immediately before use. Do not vortex enzyme. Prolonged storage of diluted enzyme or vigorously vortexing will denature the enzyme.

- Test compound: Dilute test compounds with deionized water or an appropriate vehicle.

- Control inhibitor: Ac-DEVD-CHO (Component C) is a caspase-3/7 inhibitor. Dilute Ac-DEVD-CHO 10-fold in assay buffer.

2. Set up enzymatic reaction.

2.1 Add test compounds and caspase-3/7 diluent into microplate. The suggested total volume of test compound and caspase-3/7 diluent is 50 µL/well.

2.2 Set up the following controls at the same time:

- Positive control contains caspase-3/7 diluent without test compound.
- Inhibitor control contains caspase-3/7 diluent and a known inhibitor, Ac-DEVD-CHO (10 µL/well).
- Vehicle control contains caspase-3/7 diluent and vehicle used to deliver test compound.

2.3 Test compound control contains assay buffer and test compound. Some test compounds have strong autofluorescence and may give false results.

2.4 Substrate control contains assay buffer.

Note: Bring up the total volume of all the controls to 50 µL/well using assay buffer.

3. Pre-incubation.

3.1 Incubate the plate at the desired temperature for enzymatic reaction for 10-15 min. In the meantime, also incubate the caspase-3/7 substrate solution at the same temperature.

4. Initiate the enzymatic reaction.

4.1 Add 50 µL/well of caspase-3/7 substrate solution into the wells. Mix the reagents completely by shaking on a plate shaker for 30-60 sec at 300-400 rpm.

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 on a plate shaker at 100-200 rpm. Keep the plate away from direct light. Measure fluorescence intensity at Ex/Em=490 nm/520 nm.

Appendix I: Data Analysis

- The fluorescence reading from the non-cell control well or substrate control well is the background fluorescence. This background reading has to be subtracted from the readings of the other wells to get the relative fluorescence unit (RFU).
- For kinetics reading:
  - Plot data as RFU versus time for each sample.
  - Determine the range of initial time points during which the reaction is linear. 10-15% conversion appears to be the optimal range.

- Obtain the initial reaction velocity (Vo) in RFU/min. Determine 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 reading:
  - Plot data as RFU versus the concentration of test compounds.
  - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, etc.

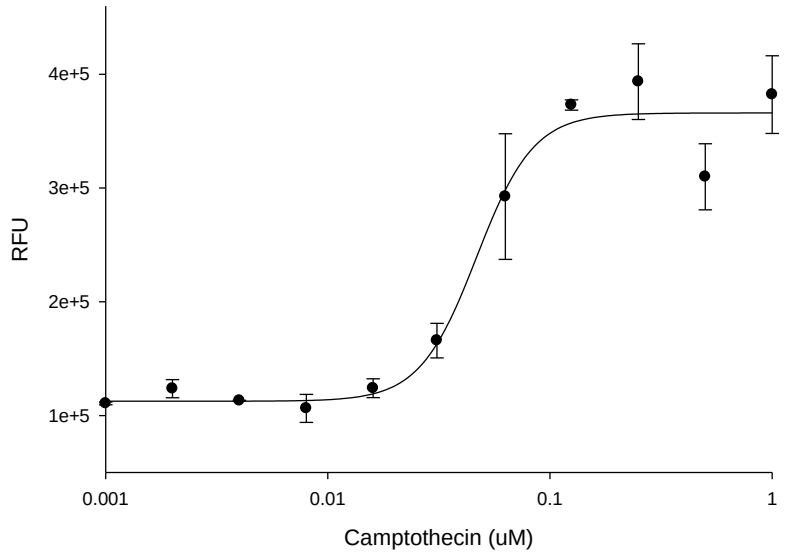


Figure 1. Dose-response curve of Camptothecin.

$1X10^5$/well Jurkat cells were treated with Camptothecin for 5h. 50 µL/well of Rh110 caspase substrate solution was added to apoptotic cells and incubated at room temperature for 30 min. Endpoint fluorescence signal was measured by a fluorescence microplate reader (FlexStation II384, Molecular Device, CA) with Ex/Em=490 nm/520 nm, cut-off 515 nm. EC_{50}= 0.047±0.006 µM.

Appendix II: Instrument Calibration

- Rh110 fluorescence reference standard: Add 8 µL Rh110 (1 mM, Component B) to 392 µL assay buffer to get 20 µM Rh110. Then take 200 µL of 20 µM Rh110 and add to 200 µL of deionized water to get 10 µM Rh110. Repeat the above 2-fold serial dilutions to obtain 5, 2.5, 1.25, 0.625 and 0.3125 µM Rh110 solutions, include an assay buffer blank. Add 50 µL/well of these serially diluted Rh110 solutions.
- Add 50 µL/well of caspase-3/7 substrate solution (refer to Protocol B step 1 for preparation). Mix the reagents by shaking the plate gently for 3 to 5 seconds.

Note: The caspase-3/7 substrate solution should be added into the reference standard to normalize for the fluorescence inner filter effect.

- Measure the fluorescence intensity of the reference standard and substrate control wells at Ex/Em=496 nm/520 nm. Adjust the sensitivity of the microplate reader until satisfactory signals can be read. Use the same sensitivity setting in the enzymatic reaction in protocols A and B.
- The fluorescence reading from the wells containing 0 μM Rh110 solution is the background fluorescence. This background fluorescence has to be subtracted from the readings of the other wells to get the relative fluorescence unit (RFU).
- Plot Rh110 fluorescent reference standard as RFU (relative fluorescent unit) versus concentration.

  Note: The final concentration of Rh110 reference standard solutions are 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16 and 0 μM. The resulting reference standard curve is used to calibrate for the variation of the different instruments and for the different batches of experiments. Since the proteolytic cleavage of $(Z\text{-}DEVD)_2$-Rh110 has two steps, first to Z-DEVD-Rh110 and then to Rh110, and both the intermediate and final products have fluorescence, the Rh110 reference standard **can not** serve as an indicator of the amount of caspase enzymatic reaction final product.

Appendix III: Prepare cell extract if culturing cells in plates larger than 96-well plates, e.g., 6-well plate or 10 cm plate

- Seed an appropriate amount of cells on the plate. Add apoptosis-inducing test compounds to the cells. Culture cells in a 37°C incubator for the desired exposure time.
- Set up the following controls at the same time.
- Positive control contains cells and a known apoptosis inducer.
- Negative control contains cells but no test compounds.
- Vehicle control contains cells and the vehicle used to deliver test compounds.
- Non-cell control contains growth medium but no cells.
- Test compound control contains growth medium and test compound. Some test compounds have strong autofluorescence and may give false positive results.
- Prepare 1X lysis buffer by adding 1 mL of 10X lysis buffer (Component F) to 9 mL of deionized water.
- Suspension cells are collected by centrifugation at 500 X g for 5 minutes. For adherent cells, simply aspirate the growth medium.
- Add an appropriate amount of lysis buffer to cells or cell pellet, e.g. 300 μL 1X lysis buffer for one well of 6-well plate. Scrape off the adherent cells or resuspend the cell pellet, and then collect the cell suspension in a micro centrifuge tube.
- Rotate the cell suspension on a rotating apparatus for 30 min at 4°C.
- Centrifuge the cell suspension at 2,500 X g for 10 min at 4°C.
- Collect the supernatant (supernatant may be stored at -80°C for future assay).
- Add 150 μL/well (96-well plate) of supernatant and controls.
- Continue to **Step 2 in protocol A** for caspase-3/7 assay.

References

1. Thornberry, NA. and Y. Lazebnik, *Science* **281**, 1312-1316 (1998).
2. Reed, JC. *J. Clin. Oncol.* **17**, 2941-2953 (1999).