SensoLyte® 520 Granzyme A Activity Assay Kit *Fluorimetric*

Revision number: 1.2 *Last updated: May 2019*

Catalog #	**AS-72260**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect granzyme A enzyme activity.
- ***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	5-FAM/QXL®-520 granzyme A substrate, Ex/Em=490/520 nm upon cleavage	2.0 mM, 50 µL
Component B	5-FAM, fluorescence reference standard, Ex/Em=490/520 nm	2.0 mM, 10 µL
Component C	2X Assay Buffer	20 mL
Component D	Inhibitor	10 mM, 10 µL

Other Materials Required (but not provided)

- Granzyme A source: Active enzyme (Enzo, Cat#), *E. coli.*
- 96-well microplate: Black, flat-bottom, 96-well plate with non-binding surface.
- 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 moisture.
- Component C can be stored at room temperature for convenience.

Introduction

Granzyme A (GzmA) is the most abundant serine protease secreted by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. Granzyme A enters pathogen-containing and tumor cells by the perforin pathway and induces caspase-independent apoptosis. Thus, cells that are resistant to the caspases or Granzyme B (a chief apoptotic mediator) might be sensitive to Granzyme A[1-5]. Recent studies showed that Granzyme A activates a novel programmed cell death pathway that begins in the mitochondrion[4].

Granzyme A also targets several important nuclear proteins for degradation including histones and DNA damage repair proteins. Granzyme A has proinflammatory activity inducing certain cytokines secretion. Its proinflammatory effect remains to be further investigated.

The SensoLyte® 520 Granzyme A Assay Kit provides a convenient assay for screening of enzyme inhibitors or for detecting the activity of enzyme using a novel internally quenched 5-FAM/QXL® FRET substrate. Upon cleavage by granzyme A, the FRET substrate will be separated into two fragments resulting in the release 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 components in biological samples and test compounds.

Protocol

Note 1: To prepare a 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 10 mL of 2X assay buffer (Component C) to 10 mL of deionized water.

1.2 Granzyme A substrate solution: Dilute granzyme A substrate (Component A) 100-fold in 1X assay buffer (step 1.1). Refer to Table 1. For each experiment, prepare fresh substrate solution. Table 1. Granzyme A substrate solution for one 96-well plate (100 assays)

Components	Volume
Granzyme A substrate (Component A)	50 µL
1X assay buffer (step 1.1)	4.95 mL
Total volume	5.0 mL

1.3 Granzyme A enzyme solution: Dilute the enzyme to an appropriate concentration in 1X assay buffer (step 1.1).

Note: ***Recommended amount of enzyme is 50 ng/well.*** Prepare enzyme diluents immediately before use. Do not vortex enzyme solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store enzyme solution on ice.

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34801 Campus Drive • Fremont, CA 94555 • 510.791.9560 • Fax 510.791.9572
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1.1 Granzyme A inhibitor, (3,4-Dichloroisocoumarin): Dilute the 10 mM inhibitor solution (Component D) 100-fold in 1X assay buffer (step 1.1) to get 100µM diluted inhibitor solution. Add 10 µl of the diluted inhibitor solution into each of the inhibitor control well.

2. Set up the enzymatic reaction.

2.1 Add test compounds and granzyme A enzyme solution (step 1.3) to the microplate wells. The suggested volume of enzyme solution for one well of a 96-well plate is 40 µL and test compound is 10 µL.

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

- Positive control: Add 40 µL granzyme A enzyme solution (step 1.3) and 10µL 1X assay buffer (step 1.1).
- Inhibitor control: Add 40 µL granzyme A enzyme solution (step 1.3) and 10µL granzyme A inhibitor (step 1.4).
- Vehicle control: Add 40 µL granzyme A enzyme solution (step 1.3) and 10µL vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control: Add 40µL 1X assay buffer (step 1.1) and 10µL test compound. Some test compounds have strong autofluorescence and may give false results.
- Substrate control: Add 50µL 1X assay buffer (step 1.1).

2.3 The total volume of all controls should be 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. Detect granzyme A enzymatic activity.

3.1 Add 50 µL of the granzyme A substrate solutions (step1.2) 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 no more than 30 sec.

3.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence at Ex/Em=490 nm/520 nm, at 37°C, continuously and record data every 5 min for 30 to 60 min.
- For end-point reading: Incubate the reaction at 37°C for 30 to 60 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

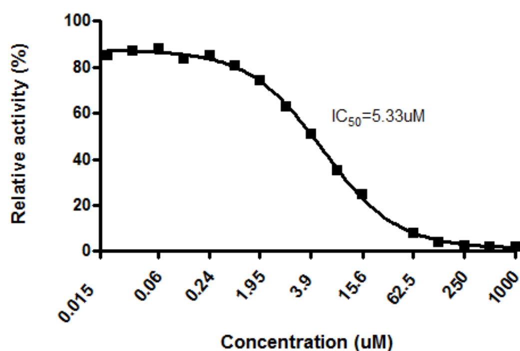

Figure 1.
Inhibition of granzyme A activity by 3,4-Dichloroisocoumarin measured with SensoLyte® 520 Granzyme A Assay Kit. (SpectraMax M5 Microplate Reader, Molecular Devices)

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

Protocol B. Measuring granzyme A activity in biological samples.

1. Prepare working solutions.

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

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

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

Components	Volume
Granzyme A (100X, Component A)	50 µL
2X Assay buffer (Component C)	4.95 mL
Total volume	5.0 mL

1.2 Granzyme A enzyme solution: If you use purified granzyme A enzyme as a positive control, then dilute the enzyme to an appropriate concentration in 1X assay buffer (Refer to Protocol A, step 1.1 for preparation). The suggested volume of enzyme solution for positive control is 50 µL/well (96-well plate).

Note 1: Mix the enzyme diluent gently. Vigorous vortexing will denature the enzyme. Keep the enzyme on ice before use.
Note 2: For positive control use substrate solution diluted in 1X assay buffer as described in Protocol A, step 1.2.

2. Set up the enzymatic reaction.

2.1 Add 50 µL of granzyme A containing biological sample.

Note: Tissue extracts and cell lysates can be prepared with assay buffer provided in the kit

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

- Positive control: Add 50µL granzyme A enzyme solution (step 1.2).
- Substrate control : Add 50µL deionized water.

2.3 The total volume of all controls should be 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. Detect granzyme A enzymatic activity

3.1 Add 50 µL of granzyme A substrate solution (step1.1) into each well. For best accuracy, it is advisable to have substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently no more than 30 sec.

3.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence at Ex/Em=490 nm/520 nm, at 37°C, continuously and record data every 5 min for 30 to 60 min.
- For end-point reading: Incubate the reaction at 37°C for 30 to 60 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

3.3 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 (optional)

- Fluorescence reference standard: Dilute 2 mM 5-FAM (Component B) 100-fold to 20 µM with 1X assay buffer (Refer to Protocol A, step 1.1 for preparation). Do 2-fold serial

dilutions to get concentrations of 10, 5, 2.5, 1.25, 0.625 and 0.3125 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 granzyme A substrate solution (refer to the protocol for preparation).

  Note: Granzyme A substrate solution is added to the 5-FAM reference standard to correct for the absorptive quenching by the FRET peptide. If multiple concentrations of substrate are used, this step must be performed for each concentration.

- Measure the fluorescence of the reference standard and substrate control wells at Ex/Em=490nm/520nm. Use the same setting of sensitivity and temperature as 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 10, 5, 2.5, 1.25, 0.625 and 0.3125 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.

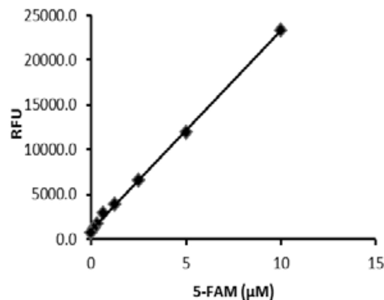

Figure 2. 5-FAM reference standard. 5-FAM was serially diluted in assay buffer containing substrate, and the fluorescence recorded at Ex/Em=490 nm/520 nm. (SpectraMax M5 Microplate Reader, Molecular Devices).

References

1. Ewen, CL. et al. (2012). A quarter century of granzymes. *Cell Death and Differentiation.* **19**, 28–35
2. Martinvalet D1, Zhu P, Lieberman J. (2005). Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis. *Immunity.* **22**(3):355-70.
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4. Lieberman J. (2010). Granzyme A activates another way to die. *Immunol Rev.,* **235** (1):93-104.
5. Beresford PJ, Xia Z, Greenberg AH, Lieberman J. (1999). Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. *Immunity.* **10**(5)*:*585-94.

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