SensoLyte® 520 Granzyme B Activity Assay Kit *Fluorimetric*

Revision number: 1.1 *Last updated: August 2024*

Catalog #	AS-72261
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect granzyme B enzyme activity.
- ***Enhanced Value:*** It provides ample reagents to perform 100 assays in a 96-well plate 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	5-FAM/QXL®-520 granzyme B substrate, Ex/Em=490/520 nm upon cleavage	0.4 mM, 50 µL
Component B	5-FAM, fluorescence reference standard, Ex/Em=490/520 nm	0.4 mM, 10 µL
Component C	Recombinant human granzyme B	10 µL
Component D	2X Assay Buffer	20 mL
Component E	Inhibitor	5 mM, 10 µL

Other Materials Required (but not provided)

- 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, except for Component C.
- Store Component C at -80°C. Aliquot as needed to avoid freeze-thaw cycles.
- Protect Components A and B from light and moisture.
- Component D can be stored at room temperature for convenience.

Introduction

Granzyme B (GzmB) is a serine protease mostly secreted by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells which are part of the innate immune system. Granzyme B is one of the major mediators of caspase-dependent apoptosis. It is released from immune cells with perforin that facilitates targeting of cells through pore forming function in their plasma membrane[1]. Recent studies showed that granzyme B can also be secreted by various non-cytotoxic cells and involved in degradation of extracellular matrix proteins thereby inducing inflammation and impairing wound healing.[2] Thus, if intracellular activity of granzyme B is mainly related to apoptosis, its extracellular activity has been linked to roles in arthritis, vascular pathologies, and other diseases.[2-4]

The SensoLyte® 520 Granzyme B Assay Kit provides a convenient assay for screening of enzyme inhibitors or for continuous assay of granzyme B activity using a novel internally quenched 5-FAM/QXL® FRET substrate. Upon cleavage by granzyme B, the FRET substrate will be separated into two fragments resulting in 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. This assay can detect as low as 0.1 ng/mL active granzyme B.

Protocol

Note 1: Please use protocol A or B based on your needs.
Note 2: Keep enzyme (Component C) on ice before use. Warm up the rest of kit components at room temperature until thawed before starting the experiments. Spin down all the vials before opening them to ensure retrieval of adequate volume of liquid in the vials.

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. Component C should be kept on ice after thawing.

1.1 1X assay buffer: Add 10 mL of 2X assay buffer (Component D) to 10 mL of deionized water.

1.2 Granzyme B substrate solution: Dilute granzyme B substrate (Component A) 100-fold in 1X assay buffer (step 1.1) according to Table 1. For each experiment, prepare fresh substrate solution

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

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

1.3 Granzyme B enzyme solution: Dilute granzyme B enzyme (Component C) 400-fold in 1X assay buffer (step 1.1). 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. Note: Prepare enzyme solution immediately before use. Do not vortex the enzyme solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store the enzyme solution on ice.

1.4 Granzyme B inhibitor, (Ac-IEPD-CHO): Dilute the 5 mM inhibitor solution (Component E) 100-fold in 1X assay buffer (step 1.1) to get 50μM 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 B 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 B enzyme solution (step 1.3) and 10μL 1X assay buffer (step 1.1).
- Inhibitor control: Add 40 μL granzyme B enzyme solution (step 1.3) and 10μL granzyme B inhibitor (step 1.4).
- Vehicle control: Add 40 μL granzyme B 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 B enzymatic activity.

3.1 Add 50 μL of the granzyme B substrate solutions (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 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.

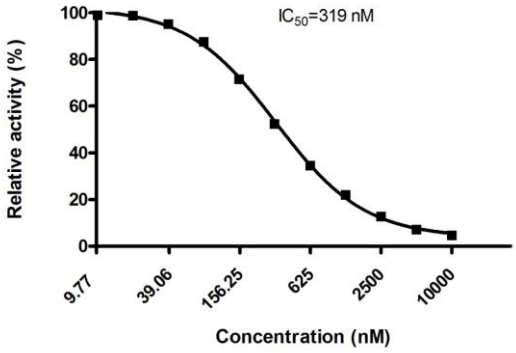

Figure 1. Inhibition of granzyme B activity by Ac-IEPD-CHO measured with SensoLyte® 520 Granzyme B Assay Kit. (SpectraMax M5 Microplate Reader, Molecular Devices)

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

Protocol B. Measuring granzyme B activity in biological samples.

1. Prepare working solutions.

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

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

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

1.2 Granzyme B enzyme solution: If you use purified granzyme B enzyme as a positive control, then dilute the enzyme (Component C) 1:500 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 solution 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 B containing biological sample.

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

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

- Positive control: Add 50µL granzyme B 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 B enzymatic activity.

3.1 Add 50 µL of granzyme B 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 0.4 mM 5-FAM (Component B) 100-fold to 4 µM with 1X assay buffer (Refer to Protocol A, step 1.1 for preparation). Do 2-fold serial

dilutions to get concentrations of 2, 1, 0.5, 0.25, 0.125 and 0.063 μM, include assay buffer blank. Add 50 μL/well of these serially diluted 5-FAM reference solutions.

- Add 50 μL/well of the diluted granzyme B substrate solution (refer to the protocol for preparation).

  Note: Granzyme B 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 2, 1, 0.5, 0.25, 0.125, 0.063, 0.031, 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.

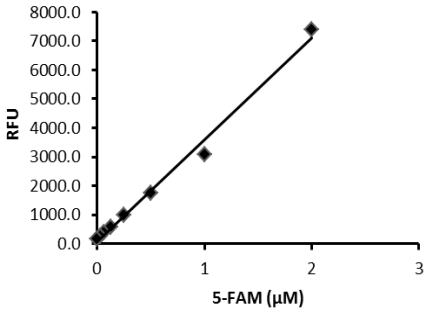

Figure 2. 5-FAM reference standard. 5-FAM was serially diluted in assay buffer containing substrate, and the fluorescence recorded at Ex/Em=490 nm/520 nm. (SpectraMax M5 Microplate Reader, Molecular Devices).

References

1. Ewen, CL. et al. (2012). A quarter century of granzymes. *Cell Death and Differentiation.* **19**, 28–35
2. Wensink, A.C.et al. (2015). Granzymes Regulate Proinflammatory Cytokine Responses. *J Immunol.* **194** (2) 491-497
3. Darrah, E. et al. (2010). Granzyme B cleavage of autoantigens in autoimmunity. *Cell Death and Differentiation.* **17** (4): 624–32.
4. Boivin, WA.et al. (2009). Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma. *Laboratory Investigation: A Journal of Technical Methods and Pathology*. **89** (11): 1195–220.

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