Technical Bulletin

Granzyme B Activity Assay Kit

Catalog Number MAK176

Product Description

cytotoxic T lymphocyte (CTL)-mediated DNA fragmentation and apoptosis in target cells. Granzyme B, a serine protease, cleaves proteins after aspartic acid residues. Granzyme B-mediated release of mitochondrial cell death factors are required for the activation of caspase-3.[1] Furthermore, the degradation of granzyme B is known to decrease the susceptibility of cancer cells to NK-cell-mediated lysis during hypoxia.[2] Granzyme B activity assays can be used for studying T cell immunity, CTL responses, cell-mediated cytotoxicity, and apoptosis.

The Granzyme B Activity Assay Kit provides a rapid, reliable, and sensitive procedure for measuring granzyme B activity in a variety of samples. Granzyme B activity is determined using an enzyme-catalyzed reaction in which the non-fluorescent substrate, Ac-IEPD-AFC, is hydrolyzed resulting in the release of AFC (λ_{Ex} = 380 nm/λ_{Em} = 500 nm) proportional to the enzymatic activity present. One unit of granzyme B is the amount of enzyme that hydrolyzes 1 pmol of the substrate per minute at 37 °C.

Components

The kit is sufficient for 100 assays in 96-well plates.

- Granzyme B Assay Buffer 25 mL
  Catalog Number MAK176A
- Granzyme B Substrate 0.5 mL
  Catalog Number MAK176B
- Granzyme B Positive Control 1 vial
  Catalog Number MAK176C
- AFC Standard, 1 mM 0.1 mL
  Catalog Number MAK176D

Reagents and Equipment Required but Not Provided

- 96-well flat-bottom plate – It is recommended to use black plates with clear bottoms for fluorescence assays. Cell culture or tissue culture treated plates are **not** recommended.
- Fluorescence multiwell plate reader

Precautions and Disclaimer

For Research Use Only. Not for use in diagnostic procedures. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice. Store components at 2–8 °C, protected from light.

Preparation Instructions

Briefly centrifuge vials prior to opening. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

Granzyme B Assay Buffer: Allow buffer to come to room temperature before use.

Granzyme B Positive Control: Reconstitute with 20 µL of Granzyme B Assay Buffer. Mix well by pipetting (Do Not Vortex), then store in single-use aliquots, protected from light, at -20 °C. Use within 1 month of reconstitution.

Procedure

All samples and standards should be run in duplicate.

Sample Preparation

Tissue (50 mg) or cells (1×10^6) can be homogenized in 200 µL of ice-cold Granzyme B Assay Buffer. Centrifuge the samples at 13,000 × g for 10 minutes to remove insoluble material.

Serum samples can be directly added to wells.

Note: For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

For all samples, bring samples to a final volume of 50 µL per well with Granzyme B Assay Buffer.

Positive Control

Add 2 µL of the Positive Control into the wells and adjust to a final volume of 50 µL per well with Granzyme B Assay Buffer.

Standard Curve Preparation

1. Prepare a 10 µM (10 pmol/µL) AFC Standard by diluting 10 µL of the 1 mM AFC Standard with 990 µl of the Granzyme B Assay Buffer.
2. Prepare AFC Standards according to Table 1. Mix well.

Table 1.
Preparation of Resorufin Standards

Well	10 µM AFC Standard	Granzyme B Assay Buffer	AFC (pmol/ well)
1	0 µL	100 µL	0
2	5 µL	95 µL	50
3	10 µL	90 µL	100
4	15 µL	85 µL	150
5	20 µL	80 µL	200
6	25 µL	75 µL	250

Assay Reaction

1. Set up the Reaction Mix according to the scheme in Table 2. 50 µL of the Reaction Mix is required for each Sample and Positive Control well.

Table 2.
Reaction Mix

Reagent	Volume
Granzyme B Assay Buffer	45 µL
Granzyme B Substrate	5 µL

2. Add 50 µL of the Reaction Mix to each of the Sample and Positive Control wells. Mix well using a horizontal shaker or by pipetting.
3. Incubate the plate at 37 °C. After 2-3 minutes, take the initial measurement (T_1). Measure the fluorescence intensity (RFU_1) using λ_{Ex} = 380 nm / λ_{Em} = 500 nm.

   Note: It is essential the initial reading (RFU_1) is within the linear range of the Standard curve.

4. Continue to incubate the plate at 37 °C taking measurements (RFU) every 5 minutes. Protect the plate from light during the incubation.
5. Continue taking measurements until the value of the most active Sample is greater than the value of the highest Standard (250 pmol/well). At this time the most active Sample is near or exceeds the end of the linear range of the Standard curve.
6. The final measurement (RFU_2) for calculating the enzyme activity would be the penultimate reading or the value before the most active Sample is near or exceeds the end of the linear range of the Standard curve (See Step 5). The time at which this reading is taken is designated T_2.

   Note: It is essential the final measurement (RFU_2) falls within the linear range of the Standard curve.

Results

Calculations

1. Correct for the background by subtracting the final measurement (RFU_2) obtained for the 0 (blank) AFC Standard (Well 1) from the final measurement (RFU_2) of each of the Standards and Samples. Background values can be significant and must be subtracted from all readings.
2. Plot the AFC Standard curve using the corrected (RFU_2) values.

   Note: A new Standard curve must be set up each time the assay is run.

3. Calculate the change in Fluorescent measurement (ΔRFU) from T_1 to T_2 for each of the Samples.

$$\Delta RFU = RFU_2 - RFU_1$$

4. Compare the ΔRFU of each Sample to the Standard curve to determine the amount (B) of AFC generated by the Granzyme B assay between T_1 and T_2.

5. Calculate the Granzyme B activity of the Sample by the following equation:

   Granzyme B Activity (pmol/min/mL) = μU/mL =

$$\frac{B \times DF}{\Delta T \times V}$$

where

B = Amount (pmol) of AFC generated between T_1 and T_2.
ΔT = Reaction time $T_2 - T_1$ (minutes)
V = Sample volume (mL) added to well
DF = Dilution factor of the Sample. DF = 1 for undiluted Samples

One unit of Granzyme B is the amount of enzyme that will generate 1.0 pmol of AFC from the substrate per minute at 37 °C.

Example

AFC amount (B) = 5,840 pmol
First reading (T_1) = 3 minutes
Second reading (T_2) = 32 minutes
Sample volume (V) = 0.05 mL
Sample dilution is 1

For this example, Granzyme B activity is:

$$\frac{5{,}840 \times 1}{(32-3) \times 0.05} = 4028\ \mu U/mL$$

References

1. Lord, S.J. et al., Granzyme B: a natural born killer. *Immunol. Rev*., **193**, 31-38 (2003).
2. Lindner, S., et al., Interleukin 21-induced granzyme B-expressing B cells infiltrate tumors and regulate T cells. *Cancer Res*., **73(8)**, 2468-2479 (2013).

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Cold assay buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96 well plate used	For Fluorometric assays, use black plates with clear bottoms
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Use of improperly stored reagents	Check the storage temperature prior to using the kit and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh Master Reaction Mix before each use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the Reaction Mix	Prepare a Master Reaction Mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the plate well
	Standard stock is at incorrect concentration	Refer to the standard dilution instructions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

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