ELISA PRODUCT INFORMATION & MANUAL

Granzyme B Activity Assay Kit (Fluorometric)

NBP2-54853

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only.
Not for diagnostic or therapeutic procedures.

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1. Overview

Granzyme B Activity Assay Kit (Fluorometric) (NBP2-54853) provides a simple and sensitive assay to detect activity of Granzyme B in cell lysates. The assay is based on the ability of Granzyme B to cleave a synthetic AFC-based peptide substrate (Ac-IEPD-AFC, which contains Granzyme B cleavage recognition sequence) to release AFC, which can be easily quantified using a fluorescence microplate reader at Ex/Em = 380/500 nm.

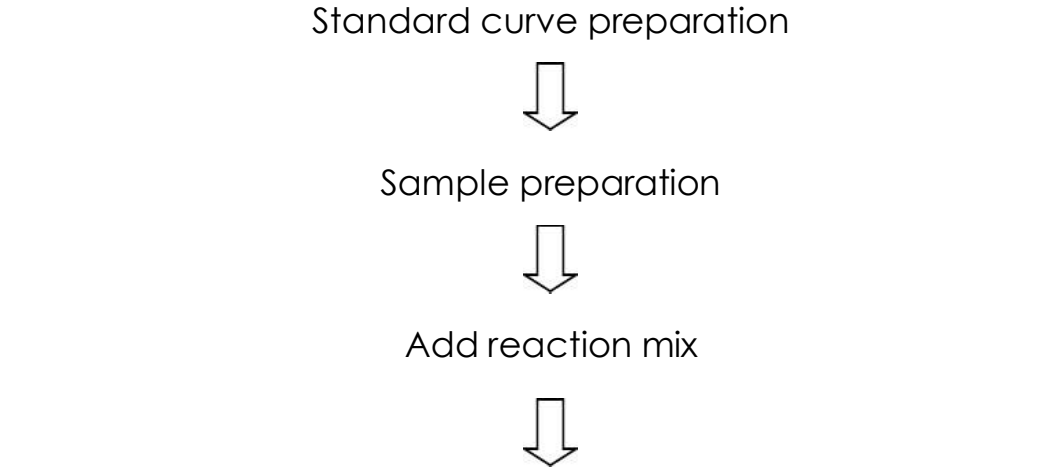
This kit can be used to quantify Granzyme B activity from cell lysates or purified Granzyme B protein, as well as to study/characterize Granzyme B inhibitors.

AFC- Substrate $\xrightarrow{\text{Granzyme B}}$ Cleaved substrate + AFC
(fluorescence at Ex/Em = 380/500 nm)

Granzyme B (Granzyme-2, GZMB, E.C. 3.4.21.79) is a serine protease most commonly found in the granules of cytotoxic lymphocytes (CTLs), NK cells and cytotoxic T cells. Upon target cell contact, Granzyme B is secreted along with perforin, a pore forming protein that mediates entrance of Granzyme B in the target cell. Granzyme B mediates apoptosis in target cell by processing and activating the initiator caspases 8 and 10, and executioner caspases 3 and 7. Caspase 7 is the most sensitive to Granzyme B.

Granzyme B contains a catalytic triad histidine-aspartic acid-serine in its active site and preferentially cleaves after an aspartic acid residue situated in the P1 site of the substrate.

2. Protocol Summary



**For kinetic mode detection, incubation time given in this summary is for guidance only*

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

Δ Note: Reconstituted component is stable for 1 month.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition (Before prep)	Storage Condition (After prep)
Granzyme B Assay Buffer	25 mL	-20°C	-20°C
Granzyme B Substrate	0.5 mL	-20°C	-20°C
AFC Standard (1 mM)	0.1 mL	-20°C	-20°C
Positive Control (Granzyme B enzyme, human recombinant)	1 vial	-20°C	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring fluorescence at Ex/Em = 380/500 nm
- Cold PBS
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with white flat bottom, preferably black

8. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Samples generating values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Granzyme B Assay Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 Granzyme B Substrate:

Ready to use as supplied. Equilibrate to room temperature before use. Aliquot so that you have enough to perform the desired number of assays. Store at -20°C.

9.3 AFC Standard (1 mM):

Ready to use as supplied. Equilibrate to room temperature. Keep on ice while in use. Aliquot standard so that you have enough to perform the desired number of assays. Store at -20°C.

9.4 Positive Control (Granzyme B, human recombinant):

Reconstitute enzyme in 20 µL Granzyme B Assay Buffer. Mix well by pipetting up and down. Aliquot positive control so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light. Avoid freeze/thaw. Once the probe is thawed, use within one month.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

10.1 Prepare a 10 µM AFC Standard by diluting 10 µL of the 1 mM AFC Standard stock solution in 990 µL Granzyme B Assay Buffer.

10.2 Using the 10 µM AFC standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	10 µM AFC Standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End amount AFC in well (pmol/well)
1	0	300	100	0
2	15	285	100	50
3	30	270	100	100
4	45	255	100	150
5	60	240	100	200
6	75	225	100	250

Each dilution has enough amount of standard to set up duplicate readings (2 x 100 µL).

11. Sample Preparation

General sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C. When you are ready to test your samples, thaw them on ice and proceed with the Sample Preparation step. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 Cell (adherent or suspension) samples:

11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 2 x 10^6 cells).
11.1.2 Wash cells with cold PBS.
11.1.3 Homogenize cells with 500 µL ice cold Assay Buffer quickly by pipetting up and down a few times.
11.1.4 Keep on ice for 10 minutes.
11.1.5 Centrifuge sample for 10 minutes at 4°C at 10,000 x*g* using a cold microcentrifuge to remove any insoluble material.
11.1.6 Collect supernatant and transfer to a new tube.
11.1.7 Keep on ice.

11.2 Purified protein:

No preparation steps are required.

Δ Note: We suggest using different volumes of sample to ensure readings are within the standard curve range.

12. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

12.1 Plate Loading:

- Standard wells = 100 µL standard dilutions.
- Sample wells = 1 – 50 µL samples (adjust volume to 50 µL/well with Granzyme B Assay Buffer).
- Positive Control = 2 µL Positive Control + 48 µL Granzyme B Assay Buffer.

12.2 Assay Reaction:

12.2.1 Prepare 50 µL of Reaction Mix for each reaction. Mix enough reagents for the number of assays to be performed. Prepare a master mix to ensure consistency.

Component	Reaction Mix (µL)
Granzyme B Assay Buffer	45
Granzyme B Substrate	5

12.2.2 Add 50 µL of Reaction Mix into each sample and positive control wells. Mix well. DO NOT add Reaction Mix to standard wells.

12.3 Measurement:

12.3.1 Measure immediately fluorescence at Ex/Em = 380/500 nm in a microplate reader in kinetic mode for 30 – 60 minutes at 37°C protected from light.

Δ Note: Incubation time depends on the Granzyme B activity in the samples. Longer incubation time may be required if activity in the sample is low. We recommend measuring fluorescence in kinetic mode, and choosing two time points (T1 and T2) in the linear range to calculate the activity of the samples. The AFC Standard Curve can be read in endpoint mode (i.e. at the end of incubation time).

13. Calculations

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.
- Use only the linear rate for calculation.

13.1 Standard curve calculation:

13.1.1 Subtract the mean fluorescence value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.1.2 Average the duplicate reading for each standard.

13.1.3 Plot standard curve readings and draw the line of the best fit to construct the standard curve (most plate reader software or Excel can do this step). Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

13.2 Measurement of Granzyme B activity in the sample:

13.2.1 For all reaction wells, choose two time points (T1 and T2) in the linear phase of the reaction progress curves and obtain the corresponding RFU values at those points (RFU1 and RFU2)

13.2.2 Calculate ΔRFU for sample as follows:
ΔRFU = RFU2 – RFU1

13.2.3 Apply the ΔRFU to AFC Standard Curve to get B pmol of AFC generated during the reaction time.

13.3 Granzyme B activity (pmol/min/mL or U/mL) in the test samples is calculated as:

$$Granzyme\ B\ Activity = \left(\frac{B}{\Delta T x\ V}\right) * D$$

Where:

B = amount of AFC in sample well calculated from standard curve (pmol).

ΔT = linear phase reaction time T2 – T1 (minutes).

V = original sample volume added into the reaction well (mL).

D = sample dilution factor.

Unit definition:

1 Unit Granzyme B activity = amount of Granzyme B that will hydrolyze 1.0 pmol of Ac-IEPD-AFC per minute at 37°C.

14. Typical data

Typical standard curve – data provided for **demonstration purposes** only. A new standard curve must be generated for each assay performed.

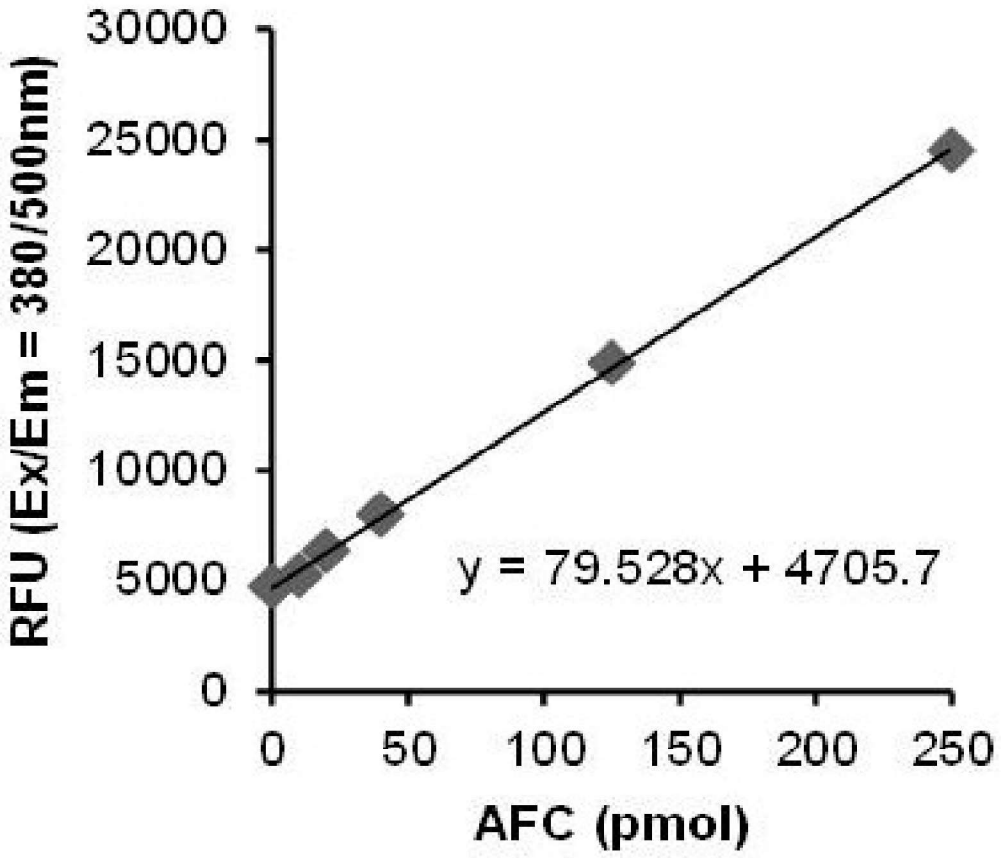


Figure 1. Typical AFC standard calibration curve.

15. Quick Assay Procedure

Δ Note: this procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare reagents (aliquot if necessary); get equipment ready.
- Prepare AFC standard dilution [50-250 pmol/well].
- Prepare samples in optimal dilutions to fit standard curve readings.
- Set up plate in duplicate for standard (100 µL), samples (50 µL) and positive control wells (50 µL).
- Prepare a master mix for Reaction Mix:

Component	Reaction Mix (µL)
Granzyme B Assay Buffer	45
Granzyme B substrate	5

- Add 50 µL Reaction Mix to sample and positive control wells. Do not add reaction mix to standard wells.
- Measure fluorescence immediately at Ex/Em= 380/500 nm in a microplate reader in a kinetic mode at 37°C for 30 – 60 minutes protected from light.

16. Troubleshooting

Problem	Reason	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at assay temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different microplate	Colorimetric: clear plates Fluorometric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates
Sample with erratic readings	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Reason	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions described in the protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. FAQs

Q. Can I use this kit in cells expressing EGFP or ECFP?

A. The kit uses an excitation and emission wavelength of Ex/Em = 380/500 nm. The excitation and emission wavelengths of EGFP and ECFP are, respectively, Ex/Em = 488/507 and 458/480 nm. Although the emission is extremely close, the fluorescent proteins should not get excited with a 380 nm wavelength.

We would recommend setting up a pilot experiment using your cell lysates only and measure fluorescence at Ex/Em = 380/500 nm to check for background.

18. Notes