Granzyme B Colorimetric Drug Discovery Kit

A complete assay system designed to screen granzyme B inhibitors.

Instruction Manual
BML-AK711

For research use only

USE FOR RESEARCH PURPOSES ONLY

Unless otherwise specified expressly on the packaging, all products sold hereunder are intended for and may be used for research purposes only and may not be used for food, drug, cosmetic or household use or for the diagnosis or treatment of human beings. Purchase does not include any right or license to use, develop or otherwise exploit these products commercially. Any commercial use, development or exploitation of these products or development using these products without the express written authorization of Enzo Life Sciences, Inc. is strictly prohibited. Buyer assumes all risk and liability for the use and/or results obtained by the use of the products covered by this invoice whether used singularly or in combination with other products.

LIMITED WARRANTY; DISCLAIMER OF WARRANTIES

These products are offered under a limited warranty. The products are guaranteed to meet all appropriate specifications described in the package insert at the time of shipment. Enzo Life Sciences' sole obligation is to replace the product to the extent of the purchasing price. All claims must be made to Enzo Life Sciences, Inc., within five (5) days of receipt of order. THIS WARRANTY IS EXPRESSLY IN LIEU OF ANY OTHER WARRANTIES OR LIABILITIES, EXPRESS OR IMPLIED, INCLUDING WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, AND NONINFRINGEMENT OF THE PATENT OR OTHER INTELLECTUAL PROPERTY RIGHTS OF OTHERS, AND ALL SUCH WARRANTIES (AND ANY OTHER WARRANTIES IMPLIED BY LAW) ARE EXPRESSLY DISCLAIMED.

TRADEMARKS AND PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending.

BACKGROUND

Granzyme B is a serine protease found in the lymphocyte granules of cytotoxic T-lymphocytes (CTLs) and natural killer (NK) cells[1]. Human granzyme B is transcribed as a pre-pro-protease. The presequence of the granzyme B peptide is removed by a signal peptidase at the endoplasmic reticulum, while it is believed that the prosequence is cleaved, and the enzyme activated, by the lysosomal cysteine protease dipeptidyl peptidase I[2]. Activated granzyme B prefers an aspartic acid residue at the P1 site of its substrates[3]; a preference shared by only one other group of mammalian proteases, the caspases. The optimal peptide sequence for granzyme B cleavage has been found to be IEPD[4,5]. Granzyme B is critically involved in the rapid apoptotic signal delivered by CTLs and NK cells[6,7,8]. *In vitro* experiments have shown that granzyme B is able to cleave and activate various caspases[9,10]. Therefore, it is believed that it delivers an apoptotic signal primarily by activating the caspase signal cascade. Other studies have shown that granzyme B is also able to cleave non-caspase proteins which are targeted during apoptosis, indicating that granzyme B may also act as an apoptotic effector[11,12].

Granzyme B plays a crucial role in the development of acute graft-vs.-host disease[13,14]. Thus, the inhibition of this enzyme may prove to be an important tool in controlling immune responses. Conversely, the failure of granzyme B-induced apoptosis may contribute to certain pathologies. Such apoptosis is important for the elimination of virus-infected and malignant cells and resistance to granzyme B action, conferred by the serpin PI-9[15], may aid in the escape of some tumors from immune surveillance[16].

The *Granzyme B Colorimetric Drug Discovery Kit* is a complete assay system designed to screen granzyme B inhibitors. It contains a colorimetric substrate (IEPD-pNA) to measure this activity; cleavage of the p-nitroanilide (pNA) group from the colorimetric substrate increases absorption at 405nm. The assays are performed in a convenient, 96-well microplate format. The kit is useful to screen for inhibitors of granzyme B, a potential therapeutic target. An inhibitor, 3, 4-Dichloroisocoumarin, is also included for use as a control.

REFERENCES:
1. M.J. Smyth and J.A. Trapani *Immunol. Today* 1995 **16** 202
2. M.J. McGuire *et al. J. Biol. Chem.* 1993 **268** 2458
3. S. Odake *et al. Biochemistry* 1991 **30** 2217
4. N.A. Thornberry *et al. J. Biol. Chem.* 1997 **272** 17907
5. J.L. Harris *et al. J. Biol. Chem.* 1998 **42** 27364
6. P.A. Henkart *Immunity* 1994 **1** 343
7. C. T. N. Pham *et al. Semin. Immunol.* 1997 **9** 127
8. J. W. Heusel *et al. Cell* 1994 **76** 977
9. A.J. Darmon *et al. Nature* 1995 **377** 446
10. M. Muzio *et al. Cell* 1996 **85** 817
11. F. Andrade *et al. Immunity* 1998 **8** 451

12. S. L. Oei *et al. Rev. Physiol. Biochem. Pharmacol.* 1997 **131** 127
13. T.A. Graubert *et al. Blood* 1996 **87** 1232
14. T.A. Graubert *et al. J. Clin. Invest.* 1997 **100** 904
15. J. Sun *et al. J. Biol. Chem.* 1996 **271** 27802
16. J.P. Medema *et al. Proc. Natl. Acad. Sci. USA* 2001 **98** 11515

PLEASE READ ENTIRE BOOKLET BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT ENZO LIFE SCIENCES TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

COMPONENTS OF BML-AK711 KIT

BML-SE238-5000 Granzyme B ENZYME (HUMAN, RECOMBINANT)
FORM: 100 U/µL in storage buffer. One U=1 pmol/min@30°C, 200 µM IEPD-pNA. Purity >90% by SDS-PAGE.
STORAGE: -80°C; AVOID FREEZE/THAW CYCLES!
QUANTITY: 5000 U (50 µL)

BML-P133-9090 SUBSTRATE (Ac-IEPD-pNA; MW=634.7)
FORM: 2 mM in Granzyme B Assay Buffer
STORAGE: -80°C
QUANTITY: 1 mL

BML-KI106-0001 CALIBRATION STANDARD (p-nitroaniline; MW=138)
FORM: 50 µM in assay buffer
STORAGE: -80°C
QUANTITY: 1 mL

BML-PI110-9090 INHIBITOR (3, 4-Dichloroisocoumarin; MW=215)
FORM: 50mM in DMSO
STORAGE: -20°C
QUANTITY: 20 µL

BML-KI121-0020 ASSAY BUFFER
(50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol)
FORM: Liquid in screw-cap plastic bottle
STORAGE: -20°C
QUANTITY: 20 mL

80-2404 ½-VOLUME MICROPLATE
1 clear, 96-well
STORAGE: Room temperature

OTHER MATERIALS REQUIRED

Microplate reader capable of measuring A_{405} to ≥3-decimal accuracy
Pipettor or multi-channel pipettor capable of pipetting 10-100 µL accurately (note: dilution of reagents can be made to increase the minimal volume to >10 µL)
Ice bucket to keep reagents cold until use

EXPERIMENTAL METHODS

Note on storage: *Store all components except the microplate at -70°C for the highest stability. The Granzyme B enzyme component, BML-SE238, must be handled with particular care in order to retain maximum enzymatic activity. Defrost it quickly in a RT water bath or by rubbing between fingers, then immediately store in an ice bath. The enzyme is stable on ice for the time typically required to set up an experiment (30-60 min.), but does lose activity with prolonged storage on ice (half-life= ~5 hrs.). It is recommended that thawing and dilution of the enzyme be done within as short a time as possible before start of the assay. The remaining, unused enzyme should be refrozen quickly by, for example, snap-freezing in a dry/ice ethanol bath or by placing it at -70°C. The enzyme is stable to at least 4 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the Granzyme B into separate tubes and store at -70°C.*

To start assay:

1. Defrost all kit components, except the enzyme, and hold on ice bath until use. The granzyme B enzyme can lose activity with prolonged storage on ice and should be handled accordingly (see storage note above). All other components are highly stable for several hours in an ice bath.
2. Dilute inhibitor (3, 4-Dichloroisocoumarin) 1/100 in assay buffer. (Note: Bring the tube of 3, 4-Dichloroisocoumarin to room temperature to thaw.) Example: Add 1 µL inhibitor to 99 µL assay buffer, in a separate tube.
3. Add appropriate volume of assay buffer to the wells of the ½-volume microplate to be used in the assay. Table I lists examples of several types of reaction (Enzyme Blank, Control, +Inhibitor) and the volume of assay buffer, and other solutions, to be used in each case. Total reaction volume is 100 µL.
4. Allow microplate to equilibrate to assay temperature (e.g. 30°C).
5. Dilute enough granzyme B, BML-SE238, 1/30 in assay buffer to produce required quantity (15 µL is needed per well). For example, dilute 10 µL granzyme B into 290 µL assay buffer.
6. Add 15 µL of granzyme B (diluted in step 3) to the "Control", "Inhibitor" and "Test Sample" wells. Final amount of granzyme B will be 50 U per well. DO NOT ADD granzyme B- TO BLANKS!
7. Add 20 µL 3, 4-Dichloroisocoumarin inhibitor (diluted in step 2) to the "Inhibitor" well only! Final inhibitor concentration=0.1 mM.
8. Add desired volume of test sample(s) to appropriate well(s). See Table 1.
9. Incubate plate for 10 min at reaction temperature (or as desired) to allow inhibitor/enzyme interaction.
10. Prepare a dilution of the substrate, Ac-IEPD-pNA, which is 2x the desired assay concentration. Fifty µl will needed for each well. For example, dilute the 2 mM stock (BML-P133-9090) to 400 µM for a final assay concentration of 200 µM. Equilibrate this 2x substrate solution to assay temperature (e.g. 30°C).
11. Start reaction by the addition of 50 µL of the 2x Ac-IEPD-pNA substrate solution.
12. Read plate continuously, at A_{405nm} in a microplate-reading spectrophotometer. For example, record data at 1 min. intervals for a total of 10 to 60 min.
13. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells!

TABLE 1. ASSAY MIXTURE EXAMPLES.

Sample	Assay Buffer	Granzyme B (3.33 U/µl)	Inhibitor	Substrate
No Enzyme Blank	**50 µL**	**0**	**0**	**50 µL**
Control	**35 µL**	**15 µL**	**0**	**50 µL**
+Inhibitor‡	**15 µL**	**15 µL**	**20 µL‡**	**50 µL**
Test sample*	**X µL**	**15 µL**	**Y µL**	**50 µL**

‡Refers to 20 µL of the diluted 3, 4-Dichloroisocoumarin prepared in step 2.

*Test sample is the experimental inhibitor. Dissolve/dilute inhibitor into assay buffer and add to appropriate wells at desired volume "Y". Adjust volume "X" to bring the total volume to 100 µL (X+Y=35 µL).

DATA ANALYSIS

PLOTTING

1. Plot data as A_{405nm} versus time for each sample.
2. Determine the time range over which the reaction is linear. Typically, 0 to 15 min works well.
3. Obtain a "best fit" line for the data points and determine the slope.
4. Average the slopes of duplicate samples.

DATA REDUCTION

5. If the blank has a significant slope, subtract this number from the slopes for all samples. Under normal circumstances this will not be necessary, since the slope will be nearly 0.
6. To find % remaining activity in presence of inhibitor:
   % activity remaining (plus inhibitor)=
   (slope of + inhibitor sample/control slope) x 100
7. To find the activity of the samples expressed as pmol substrate/min:
   Determine microplate reader conversion factor:
   a) Add 100 µL calibration standard (p-nitroaniline; 50 µM concentration) to 2 wells of the ½-volume microplate. The extinction coefficient for p-nitroaniline at 405 nm is ~10,500 $M^{-1}cm^{-1}$. Typically, 100 µL of the 50 µM standard, in a ½ volume well, produces an A_{405} of about 0.3 (or 300 mOD)
   b) Determine the average A_{405nm} using 100 µL assay buffer as a blank.
   c) Calculate the conversion factor.

      conversion factor (µM/mOD) =
      50 µM ÷ average A_{405} from step b

   d) Calculate the activity as pmol/min:
      activity (pmol/min) = slope (mOD/min) x conversion factor(µM/mOD) x assay vol (µL)

   The assay vol in µL = 100 for the standard assay.
   NOTE: If a different volume is used, be sure to perform steps a) to d) using the actual assay volume.

Sample activity calculation:

conversion factor = 50 µM ÷ 294 mOD = 0.170 µM/mOD

Example: sample for which the slope of the $A_{405\ nm}$ vs. time plot is 1.7 (mOD/min)
activity =
1.7 (mOD/min) x 0.170(µM/mOD) x 100(µl) = 30 pmol/min

APPLICATION EXAMPLES

The *Granzyme B Assay Kit for Drug Discovery* can be used to study enzyme regulation and kinetics, cleave target substrates and screen inhibitors of Granzyme B.

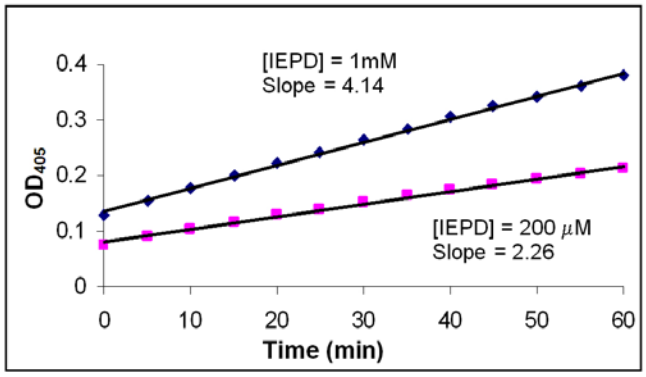

Figure 1. Granzyme B activity stable for at least 1 hour under the conditions of the IEPD-pNA cleavage assay. [IEPD-pNA]=1mM and 200 μM; 30°C

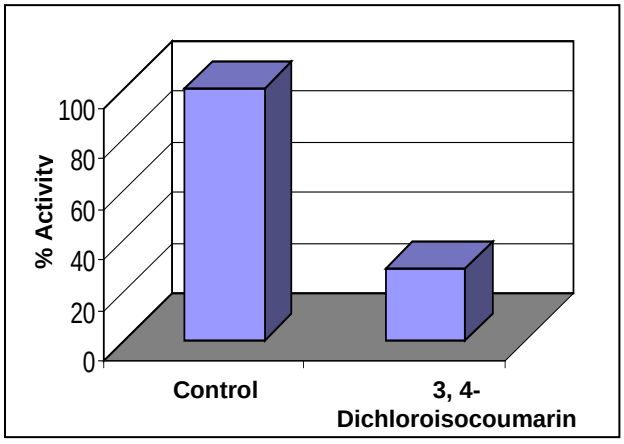

Figure 2. Inhibition of Granzyme B by 3, 4-Dichloroisocoumarin. The enzyme was incubated with the inhibitor for 30 minutes prior to addition of substrate. Granzyme B = 50 U/well; [3, 4-Dichloroisocoumarin] =100 μM; [IEPD]=200 μM; 30°C

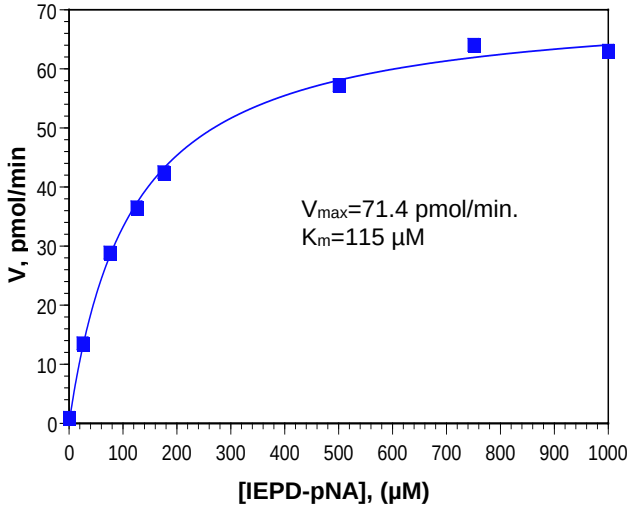

Figure3. Kinetics of IEPD-pNA cleavage by Granzyme B. 50 U/well; 30°C. Rates were obtained from the slopes of the initial, linear portion of plots of A_{405} vs. time. Curve and kinetic parameters derive from a non-linear least squares fit to the Michaelis-Menten equation (Marquadt algorithm).

www.enzolifesciences.com

Enabling Discovery in Life Science®

Global Headquarters
Enzo Life Sciences Inc.
10 Executive Blvd
Farmingdale, NY 11735
(p) 1-800-942-0430
(f) 1-631-694-7501
(e) info-usa@enzolifesciences.com

Enzo Life Sciences (ELS) AG
Industriestrasse 17, Postfach
CH-4415 Lausen / Switzerland
(p) +41/0 61 926 89 89
(f) +41/0 61 926 89 79
(e) info-ch@enzolifesciences.com

Please visit our website at www.enzolifesciences.com for additional contact information.