Product Manual

Cathepsin S Drug Discovery Kit

Designed to screen modulators of cathepsin S activity.

Catalog # BML-AK431

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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 Support if necessary.

INTRODUCTION

Cathepsin S is a member of the cathepsin family of cysteine proteases[1]. These enzymes play a role in many normal and disease states by virtue of their broad substrate specificities[2,3]. Targets of cathepsin S include MHC Class II-associated invariant chain4, laminin-5[5], amyloid beta peptide, and MBP (myelin basic protein)[6]. Cathepsin S is expressed as a 35 kDa proenzyme (as measured by SDS-PAGE), and activated by cleavage to 24 kDa[7]. It is an important target for drug discovery due to its involvement in immune system function, rheumatoid arthritis, multiple sclerosis, and cancer[4-5,8-9].

The Cathepsin S Drug Discovery Kit is a complete system designed to screen modulators of cathepsin S activity. Included are active cathepsin S enzyme, fluorogenic (Z-VVR-AMC) substrate, assay buffer, and control inhibitor E-64[10]. The assays are performed in a convenient 96-well microplate format.

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MATERIALS PROVIDED

BML-SE453-9090 Cathepsin S enzyme (human, recombinant)

FORM: Recombinant human cathepsin S, 0.290 mU/µL. One U=1µmole/min@37°C, 40 µM Z-VVR-AMC.

STORAGE: -80°C; Avoid freeze/thaw cycles QUANTITY: 8 mU (28 µL)

BML-P199-9090 AMC SUBSTRATE (Z-VVR-AMC; MW=663.8) FORM: 2 mM in DMSO

STORAGE: -80°C

QUANTITY: 220 µL

BML-KI107-0001 AMC CALIBRATION STANDARD

(7-amino-4-methylcoumarin; MW=175) FORM: 30 µM in assay buffer.

STORAGE: -80°C

QUANTITY: 1 mL

BML-PI105-9090 INHIBITOR (E-64; MW=357.4) FORM: 0.5 mM in dH2O

STORAGE: -80°C

QUANTITY: 20 µL

BML-KI431-0020 ASSAY BUFFER

50mM potassium phosphate buffer, pH 6.5, 50mM NaCl, 2mM EDTA, 0.01% Triton X-100®, 0.5mM DTT

FORM: Liquid in screw-cap plastic bottle

STORAGE: -20 or -80°C

QUANTITY: 20 mL

80-2406 ½-VOLUME WHITE NBS MICROPLATE

STORAGE: Room temperature (Sold separately as BML-KI571)

OTHER MATERIALS NEEDED

- Microplate reader capable of fluorescence at wavelengths of approximately 380nm (excitation)/ 460nm (emission).
- Pipetman or multi-channel pipetman capable of pipetting 10-100 µL accurately (note: reagents can be diluted to increase the minimal pipetting volume to >10 µL).
- Ice bucket to keep reagents cold until use.
- Water bath or incubator for component temperature equilibration.

PROTOCOL

Notes on Storage: *Store all components except the microplates (room temperature) at -80°C for the highest stability. The cathepsin S enzyme should be handled carefully in order to retain maximal enzymatic activity. It is stable, in diluted or concentrated form, for several hours on ice. As supplied, cathepsin S enzyme is stable for at least 5 freeze/thaw cycles. Nevertheless, minimize time on ice and the number of freeze/thaw cycles by aliquotting into separate tubes, storing at -80°C, and thawing shortly before use. When setting up the assay, do not maintain diluted components at reaction temperature (e.g. 37°C) for an extended period of time prior to running the assay.*

To start assay:

1. Briefly warm kit component BML-P199-9090 to RT to thaw DMSO.
2. Dilute inhibitor (E-64, BML-PI105-9090) 1/100 in assay buffer KI-431 as follows. Add 1 µL inhibitor into 100 µL assay buffer, in a separate tube. Warm to reaction temperature (e.g. 37°C). Dilute test inhibitor(s) as desired (see Table 1, below).
3. Dilute substrate BML-P199-9090 1/25 in assay buffer to required total volume (50 µL are needed per well). For example, for 15 wells dilute 32 µL P199-9090 into 768 µL assay buffer, in a separate tube. Warm to reaction temperature (e.g. 37°C).
4. Dilute Cathepsin S enzyme 1/48 in assay buffer to required total volume (10 µL are needed per well). Warm to reaction temperature (e.g. 37°C).
5. Pipet assay buffer into each desired well of the 1/2 volume microplate as follows:

   Blank (no Cathepsin S)=50 µl Assay Buffer Control (no inhibitor)=40 µL Assay Buffer Inhibitor E-64=20 µL Assay Buffer

   Test inhibitor=varies (see Table 1, below)
6. Allow microplate to equilibrate to assay temperature (e.g. 37°C).
7. Add 10 µL Cathepsin S (diluted in step 4) to the control, inhibitor E-64, and test inhibitor wells. Final amount of Cathepsin S will be 0.06 mU per well (0.6 µU/µL). Remember to **not** add Cathepsin S to blanks!

8. Add 20 µL E-64 inhibitor (diluted in step 2) to the inhibitor E-64 wells only! Final inhibitor concentration= 1 µM.
9. Add desired volume of test inhibitor to appropriate wells. See Table 1, below.
10. Incubate plate for 30-60 minutes at reaction temperature (e.g. 37°C) to allow inhibitor/enzyme interaction.
11. Start reaction by the addition of 50 µL BML-P199-9090 substrate (diluted and equilibrated to reaction temperature in step 3). Final substrate concentration= 40 µM.
12. Continuously read plates at Ex: 380 nm/Em: 460 nm in a microplate reader. Record data at 1 min. time intervals for 10 or more minutes.
13. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells.

TABLE 1. ASSAY MIXTURE EXAMPLES.

Sample	Assay Buffer	Cath. S (6 µU/µl)	Inhibitor	Substrate (80 µM)
Blank	50 µL	0	0	50 µL
Control	40 µL	10 µL	0	50 µL
Inhibitor+	20 µL	10 µL	20 µL+	50 µL
Test sample*	X µL	10 µL	Y µL*	50 µL

‡Refers to 20 µl of diluted E-64 prepared in step 2.
*Test sample is the experimental inhibitor. Dissolve/dilute experimental 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=40 µL).

Example of plate:

Well# sample
A1 Blank
B1 Blank
C1 Control
D1 Control
E1 Inhibitor E-64 F1 Inhibitor E-64 G1 Test inhibitor
H1 Test inhibitor...

DATA ANALYSIS

Plotting:

1. Plot data as Relative Fluorescence Units (RFU) versus time for each sample (see Fig. 1).
2. Determine the range of time points during which the reaction is linear. Typically, points from 1 to 10 min are sufficient.
3. Obtain the reaction velocity (**V**) in RFU/sec: determine the slope of a line fit to the linear portion of the data plot using an appropriate routine.
4. Average the slopes of duplicate samples.
5. *If the blank has a significant slope, subtract this number from all samples.*
6. To determine inhibitor % remaining activity:

Inhibitor % activity remaining = (**V** inhibitor / **V** control) x 100

See Figure 1 for example.

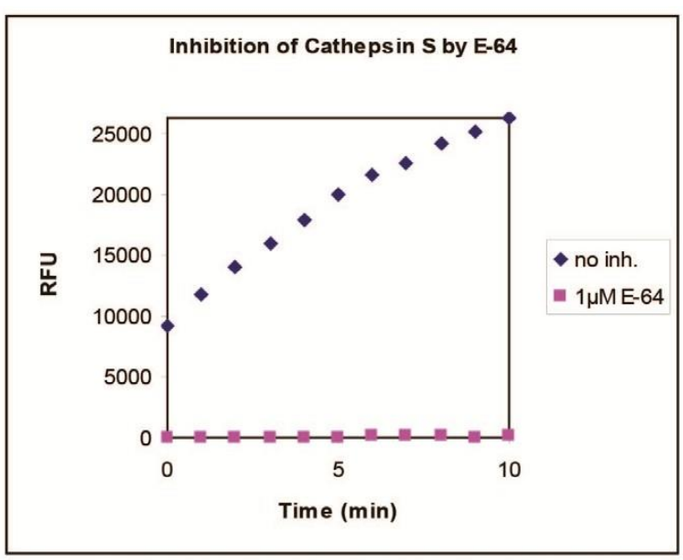
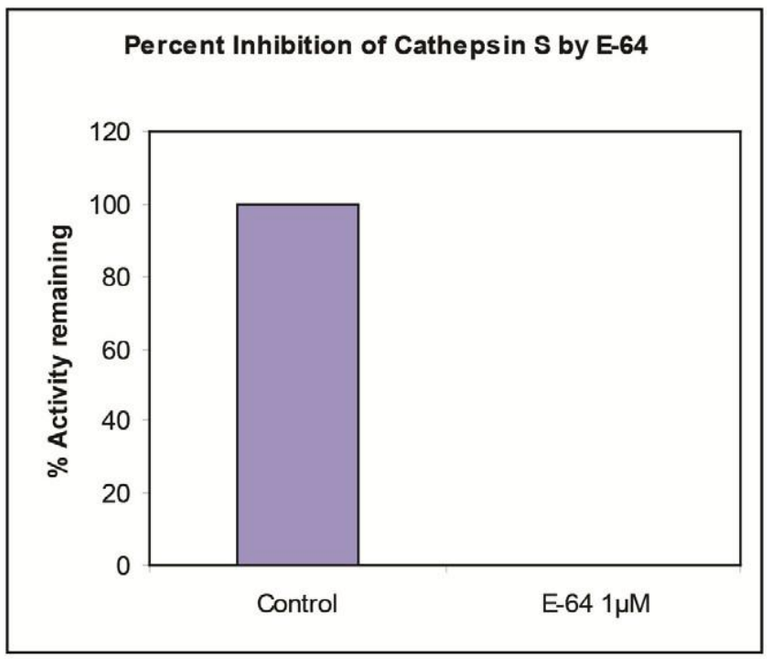


Figure 1. Example of inhibitor data

control slope = 34.28 RFU/sec

inhibitor slope = 0 RFU/sec

inhibitor % activity remaining = (0/34.28) x 100 = 0%



7. **<u>To find the activity of the samples expressed as pmol substrate/min</u>:**

a) Determine microplate reader conversion factor for AMC fluorophore. The exact AMC concentration range that will be useful for preparing a standard curve will vary depending on the fluorimeter model, the gain setting, and the exact excitation and emission wavelengths used. The AMC standard, as provided (30 µM), may yield off-scale readings in some cases. We recommend diluting some of the standard to a relatively low concentration with Assay Buffer (0.5 or 1.0 µM) and then measuring the fluorescence of 100 µL. The estimate of µM/RFU obtained with this measurement, together with the observed range of values obtained in the enzyme assays, can then be used to plan an appropriate series of dilutions for a standard curve. The slope of the standard curve can then be used as the µM/RFU conversion factor.

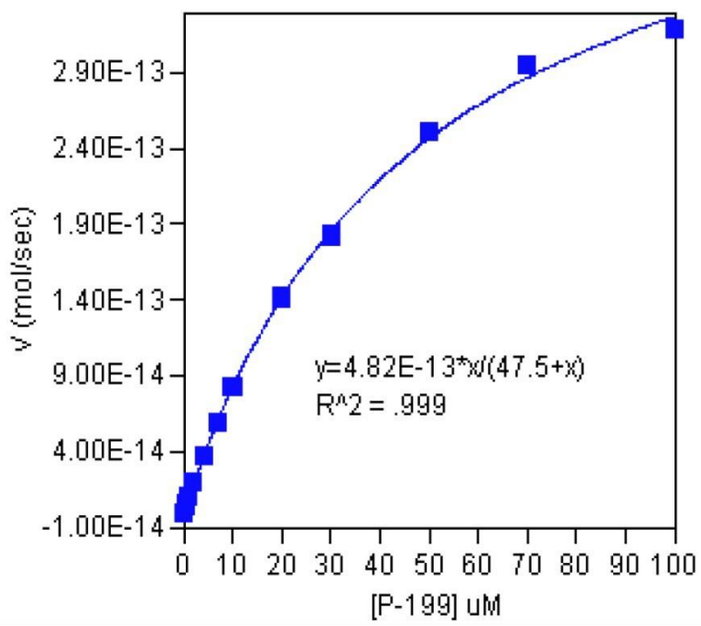
b) Calculate the activity as pmol/min:

Activity (pmol/min) = slope (RFU/sec) x 60sec/min x conversion factor (µM/RFU) x assay vol (µL)

The assay vol in µL= 100 for the standard assay.

See Figure 2 for activity and kinetic calculations.

Figure 2. Kinetics of Z-VVR-AMC cleavage by Cathepsin S, 0.059mU/well; 37°C. Rates were obtained from the slope of the initial, linear portion of plots of AFU vs. time. Curve and kinetic parameters derived from a non-linear least squares fit to the Michaelis-Menten equation (Marquadt algorithm).



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