DPPIV Drug Discovery Kit

Designed to screen DPPIV inhibitors

Instruction Manual

BML-AK499

For research use only

✦*DPPIV Drug Discovery Kit –BML- AK499*✦

BACKGROUND

DPPIV (DPP4, CD26) is a member of the class of proteases known as prolyl peptidases, which cleave proteins after proline residues[1]. DPPIV, a serine dipeptidyl peptidase, cleaves the N-terminal X-Ala or X-Pro from target polypeptides, such as chemokines (e.g. CXCL11) and peptide hormones (e.g., glucagon-like peptide-1, GLP1)[1-3]. DPPIV possesses a transmembrane region and a very short cytoplasmic domain, but is often cleaved and released as a soluble, circulating form[4]. It is found as a dimer with itself or with FAP (fibroblast activation protein-α, seprase), another prolyl peptidase[1]. It also has non-peptidase functions: through its interaction with adenosine deaminase (ADA) and extracellular matrix components, it influences T-cell activation and proliferation[2,5,6]. It is thought to play roles in diabetes, cancer, and autoimmune diseases, making it a target for drug discovery[7-11].

The *DPPIV Drug Discovery Kit* is a complete assay system designed to screen DPPIV inhibitors, providing enough material to perform at least 96 assays. The kit contains both a chromogenic substrate (H-Gly-Pro-pNA; K_m=114 µM) and a fluorogenic substrate (H-Gly-Pro-AMC; K_m=50 µM). Cleavage of the p-nitroaniline (pNA) from the chromogenic substrate increases absorbance at 405 nm. The fluorimetric assay is based on the cleavage of 7-amino-4-methylcoumarin (AMC) moiety from the C-terminus of the peptide substrate, which increases its fluorescence intensity at 460 nm. The kit is useful to screen inhibitors of DPPIV, a potential therapeutic target. A DPPIV inhibitor, P32/98 (K_I=130 nM[12]), is included for use as a control.

Other DPP enzymes are available for specificity profiling. Contact Enzo Life Sciences or go to www.enzolifesciences.com.

REFERENCES:

1. J.S. Rosenblum and J.W. Kozarich *Curr. Opin. Chem. Biol.* 2003 **7** 496
2. R. Thoma *et al. Structure* 2003 **11** 947
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10. H. Gotoh *et al. Clin. Chem.* 1988 **34** 2499
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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 SERVICES FOR ASSISTANCE IF NECESSARY.

COMPONENTS OF BML-AK499 KIT

BML-SE434-9090 DPPIV ENZYME (HUMAN, RECOMBINANT)

FORM: Recombinant soluble human DPPIV. One U=1µmole/min@37°C, 100 µM H-Gly-Pro-pNA.

STORAGE: -80°C; AVOID FREEZE/THAW CYCLES

QUANTITY: 35 mU

BML-P188-9090 pNA SUBSTRATE (H-Gly-Pro-pNA; MW=328.8)

FORM: 10 mM in DMSO

STORAGE: -80°C

QUANTITY: 150 µL

BML-KI106-0001 pNA CALIBRATION STANDARD (p-nitroaniline; MW=138)

FORM: 50 µM in assay buffer.

STORAGE: -80°C

QUANTITY: 1 mL

BML-P189-9090 AMC SUBSTRATE (H-Gly-Pro-AMC; MW=410.3)

FORM: 0.5 mM in DMSO

STORAGE: -80°C

QUANTITY: 150 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-PI142-9090 INHIBITOR (P32/98; MW=260.4)

FORM: 1 mM in DMSO

STORAGE: -80°C

QUANTITY: 20 µL

BML-KI342-0020 ASSAY BUFFER

(50 mM Tris, pH 7.5)

FORM: Liquid in screw-cap plastic bottle

STORAGE: -80°C (or +4°C)

QUANTITY: 20 mL

80-2407 ½-VOLUME CLEAR & NBS WHITE MICROPLATE – 1 EACH

STORAGE: Ambient.

OTHER MATERIALS REQUIRED

Microplate reader capable of measuring A_{405} to ≥3-decimal accuracy, or fluorescence at wavelengths of approximately 380nm (excitation)/ 460nm (emission)

Pipettes or multi-channel pipettes capable of pipetting 10-1000 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.

SUGGESTED EXPERIMENTAL METHODS

Note on storage: *Store all components except the microplates at -80°C for the highest stability. The DPPIV 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, DPPIV enzyme is stable for at least 5 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the DPPIV into separate tubes and store at -80°C. 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. Defrost kit components and hold on ice until use. Thaw and store DMSO components (substrates, inhibitor) at room temperature, preferably in a dark place. *Briefly centrifuge all vials.* Minimize the time that any kit component is thawed.
2. Dilute inhibitor (P32/98) 1/10 in assay buffer. Example: Add 2 µL inhibitor to 18 µL assay buffer, in a separate tube.
3. For colorimetric assay (at A_{405nm}, using clear microplate): Dilute substrate (H-Gly-Pro-pNA) 1/50 in assay buffer (50 µL is needed per well). Example: Add 5 µL substrate to 245 µL assay buffer, in a separate tube.
4. For fluorimetric assay (at Ex:380 nm/Em:460 nm, using white microplate), dilute substrate (H-Gly-Pro-AMC) 1/50 in assay buffer (50 µL is needed per well). Example: Add 5 µL substrate to 245 µL assay buffer, in a separate tube.
5. Add assay buffer to each desired well of microplate so that the total assay volume will be 100 µL. See **Table 1** for examples.
6. Allow microplate and diluted assay components to equilibrate to assay temperature (e.g. 37°C).
7. After a brief thawing, quickly centrifuge the vial of DPPIV (BML-SE434-9090) to bring contents to bottom of tube. Dilute enough DPPIV in assay buffer to a stock concentration of 17.3µU/µL to produce required quantity for the experiment.
8. Add DPPIV to the “Control”, “Inhibitor” and “Test Sample” wells, please note that the final amount of DPPIV *per well* is to be 0.26mU. DO NOT ADD DPPIV TO BLANKS!
9. Add 10 µL P32/98 inhibitor (diluted in step 2) to the “Inhibitor” well only! Final inhibitor concentration is 10 µM.
10. Add desired volume of test sample(s) to appropriate well(s). See **Table 1**.
11. Incubate plate for 10 min at reaction temperature (or as desired) to allow inhibitor/enzyme interaction.
12. Start assay by the addition of 50 µl H-Gly-Pro-pNA substrate (diluted in step 3) or 50 µL H-Gly-Pro-AMC substrate (diluted in step 4), which have been equilibrated to reaction temperature (e.g. 37°C). Enough of each substrate is provided to perform 96 assays at 100 µM for the chromogenic substrate or 5 µM for the fluorogenic substrate.
13. Read plate continuously, at A_{405nm} for the pNA substrate, or Ex:380 nm/Em:460 nm for the AMC substrate, in a microplate reader. For example, record data at 1 min. intervals for a total of 10 to 60 min.
14. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells.

TABLE 1. ASSAY MIXTURE EXAMPLES.

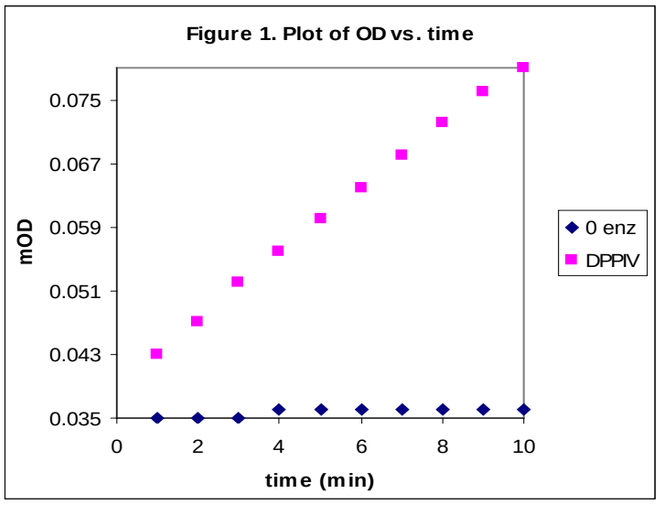
Sample	Assay Buffer	DPPIV (17.3 µU/µl)	Inhibitor	Substrate
Blank	50 µL	0	0	50 µL
Control	35 µL	15 µL	0	50 µL
Inhibitor‡	25 µL	15 µL	10 µL‡	50 µL
Test sample*	X µL	15 µL	Y µL*	50 µL

‡Refers to 10 µL of the diluted P32/98 prepared in step 2.

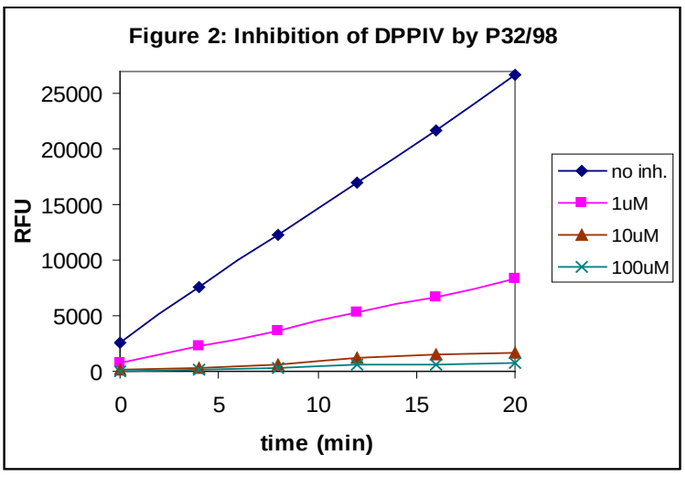
*Test sample is the experimental inhibitor. Dissolve/dilute experimental inhibitor into assay buffer and add to appropriate wells at desired volume "Y". Adjust volume "X" to bring the total volume to 100 µL (X+Y=35 µL).

DATA ANALYSIS

1. Plot data as A_{405nm} or Relative Fluorescence Units (RFU) versus time for each sample (see Figures 1 and 2).



Slope=**V**=4.04E-03 OD/min

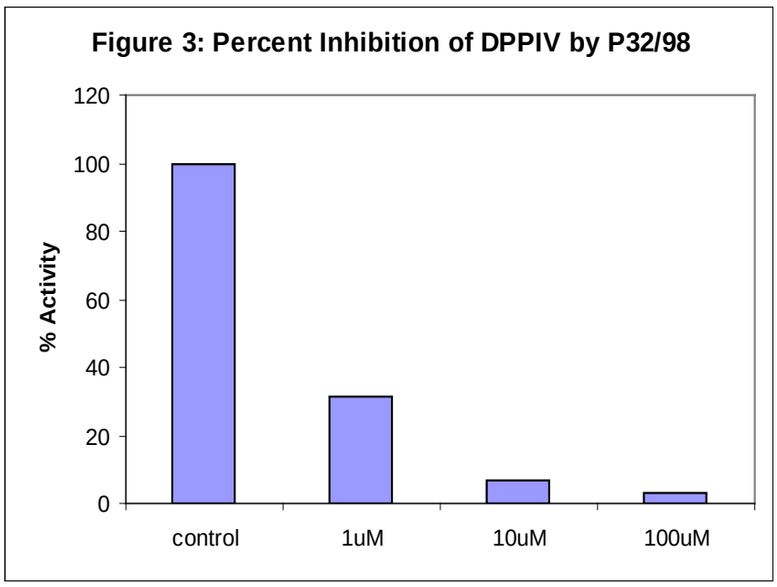


2. Determine the time range over which the reaction is linear. Typically, 0 to 15 min works well.
3. Obtain a “best fit” line for the data points and determine the slope.
4. Average the slopes of duplicate samples.
5. If the blank has a significant slope, subtract this number from the slopes for all samples.
6. <u>To find % remaining activity in presence of inhibitor</u>:

   % activity remaining (with inhibitor)=

   (slope of + inhibitor sample/control slope) x 100

   See Figure 3.



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

   Determine microplate reader conversion factor:

   a) Add 100 µL calibration standard (p-nitroaniline; 50 µM concentration) to 2 wells of the clear microplate. In Tris buffer the extinction coefficient for p-nitroaniline at 405 nm is ~9700 $M^{-1}cm^{-1}$ (R. Lottenberg et al., 1983; *Biochim.Biophys.Acta* **742**:558). Typically, 100 µL of the 50 µM standard, in a ½ volume well, produces an A_{405} of about 0.3. Alternatively, build a pNA standard curve and use the slope (µM/OD) as the conversion factor.
   b) Determine the average A_{405nm} using 100 µL assay buffer as a blank.
   c) Calculate the conversion factor.

      Conversion factor (µM/OD) =50/average A_{405} from step b)

   d) Calculate the activity as pmol/min:

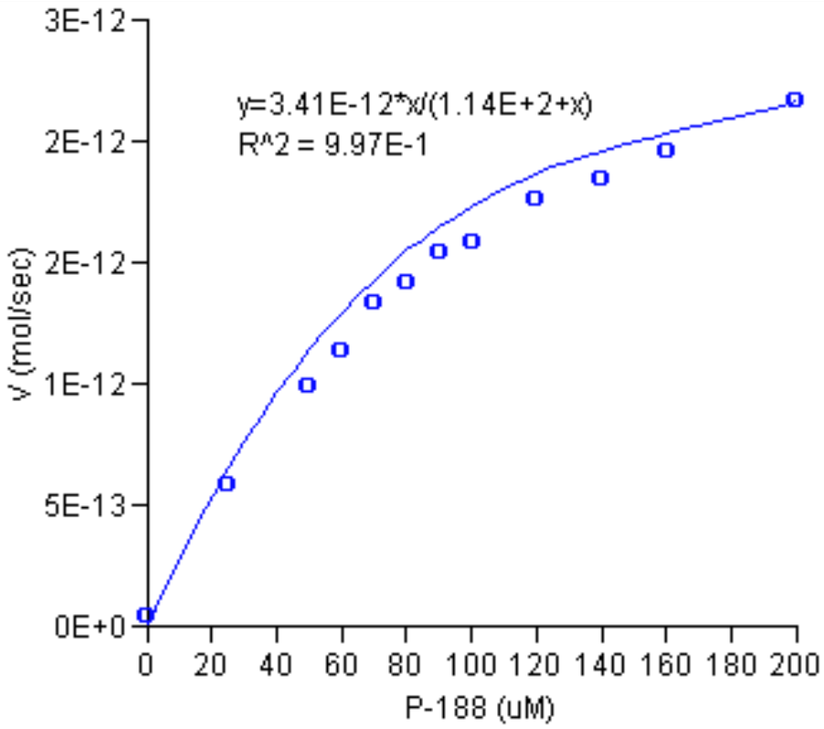
      activity (pmol/min) = slope (OD/min) x conversion factor(µM/OD) x assay vol (µL)

Example calculation for activity with colorimetric substrate:

conversion factor = 50 µM/0.294 OD = 170 µM/OD

activity of a control sample =
4.04E-03 (OD/min) x 170(µM/OD) x 100(µl) = 68 pmol/min

See Figure 4 for example of kinetic determination.



K_m=114 µM

V_{max}=3.41 pmol/sec

k_{cat}/K_m=7.66 x 10^5 $M^{-1}s^{-1}$

Figure 4. Kinetics of H-Gly-Pro-pNA cleavage by DPPIV, 0.13 mU/well; 37°C. Rates were obtained from the slopes of the initial, linear portion of plots of A_{405} vs. time. Curve and kinetic parameters derived from a non-linear least squares fit to the Michaelis-Menten equation (Marquadt algorithm).

8. To find the activity of the samples expressed as pmol substrate/min using fluorogenic substrate:
   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)

9. Inhibition kinetics:

   For determining K_i, etc., the concentration of enzyme active sites is needed. Although the concentration (as measured by the Bradford method) of DPPIV in this kit can be obtained from Enzo Life Sciences Technical Services, it is more accurate to use active site titration with an inhibitor, such as P32/98 in this kit. Please see the following references:

   a) *Active-site titration of peptidases*: C.G. Knight; Methods Enzymol. **248**, 85 (1995).
   b) *L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L:* A.J. Barrett et al.; Biochem. J. 201, 189 (1982).

NOTES

NOTES

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