DPPIV/CD26 Assay Kit for Biological Samples
Designed to measure protease activity of DPPIV/CD26

Instruction Manual
BML-AK498
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Please read entire booklet before proceeding with the assay.
INTRODUCTION

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., GLP-1, PACAP, VIP, BNP)\(^1\)\(^2\)\(^3\)\(^4\). DPPIV possesses a transmembrane region and a very short cytoplasmic domain, but is often cleaved and released as a soluble, circulating form\(^3\). It also has non-peptidase functions: through its interaction with adenosine deaminase (ADA) and extracellular matrix components, it influences T-cell activation and proliferation\(^2\)\(^5\). It is thought to play roles in diabetes, cancer, and autoimmune diseases, making it a target for drug discovery\(^2\)\(^3\)\(^6\).

Because DPPIV protein levels may not accurately reflect the levels of active DPPIV enzyme\(^3\)\(^7\), it is useful to measure DPPIV activity rather than performing DPPIV immunoassay. The DPPIV/CD26 Assay Kit for Biological Samples is a complete assay system designed to measure DPPIV activity in biological fluids such as plasma, serum, urine, and saliva (see below). Uses for the kit include correlation of DPPIV activity with disease states\(^8\)\(^9\)\(^23\)\(^29\)\(^35\)\(^40\)\(^41\), or determination of the efficacy of DPPIV inhibitors administered in vivo\(^10\)\(^13\)\(^16\)\(^18\)\(^22\)\(^43\).

The kit is validated for DPPIV assay in plasma\(^11\)\(^14\)\(^22\)\(^37\)\(^43\), serum\(^8\)\(^9\)\(^23\)\(^29\), urine\(^17\)\(^23\)\(^25\)\(^30\)\(^40\)\(^41\), and saliva\(^33\)\(^34\) (see Figures below). The kit can easily be used for other biological fluids such as tissue\(^15\)\(^17\)\(^37\); live cells and cell extracts\(^14\)\(^24\)\(^31\)\(^32\)\(^37\); and exudates\(^14\)\(^35\). Enough reagents are provided to perform at least 96 assays. The kit contains both a chromogenic substrate (H-Gly-Pro-pNA) and a fluorogenic substrate (H-Gly-Pro-AMC). 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. A specific DPPIV inhibitor, P32/98\(^13\), is included to uncover any activity not contributed by DPPIV. Also included is DPPIV enzyme to use as a positive control.

Although it can vary with disease state, DPPIV is the major (70-95%) Gly-Pro-cleaving activity in most tissues, with the exception of pancreas, colon, and brain\(^1\)\(^3\). In plasma and serum of DPPIV knockout mice, there is little or
no Gly-Pro-cleaving activity\textsuperscript{3,36,37,42}. There is no activity contributed by DPP8, DPP9, nor FAP\textsuperscript{37}. Although it cannot be ruled out that certain diseases may cause these enzymes to be present in plasma/serum\textsuperscript{3}, FAP does not efficiently cleave Gly-Pro substrates; DPP8 and DPP9 activity is unstable without glycerol added to samples; and DPP8 and DPP9 are not as efficient at cleaving Gly-Pro substrates at room temperature as is DPPIV\textsuperscript{6}. DPPII/7, which is present in plasma/serum, is not active above neutral pH\textsuperscript{38,39}.

**Assay Flow Chart**

1. Pipet Assay Buffer into Well
2. Pipet Biological Sample into Well
3. Pipet Substrate (Chromogenic or Fluorogenic) into Well
4. Read Absorbance or Fluorescence

Other DPP reagents are available. Contact Enzo Life Sciences or go to [www.enzolifesciences.com](http://www.enzolifesciences.com).

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.
MATERIALS PROVIDED AND STORAGE

**BML-SE434-9091 DPPIV ENZYME (HUMAN, RECOMBINANT)**
FORM: Recombinant soluble human DPPIV, 0.325 mU/µL. One U=1µmole/min@37°C, 100 µM H-Gly-Pro-pNA. STORAGE: -70°C; AVOID FREEZE/THAW CYCLES
QUANTITY: 5 mU (15.4 µL), sufficient for ~18 positive control wells

**BML-P188-9091 pNA SUBSTRATE** (H-Gly-Pro-pNA; MW=328.8)
FORM: 100 mM in DMSO (dimethylsulfoxide)
STORAGE: -70°C
QUANTITY: 55 µL

**BML-KI577-0600 pNA CALIBRATION STANDARD** (p-nitroaniline; MW=138)
FORM: 80 µM in 50 mM Glycine, pH 8.7, 1 mM EDTA
STORAGE: -70°C
QUANTITY: 600 µL

**BML-P189-9091 AMC SUBSTRATE** (H-Gly-Pro-AMC; MW=410.3)
FORM: 50 mM in DMSO
STORAGE: -70°C
QUANTITY: 45 µL

**BML-KI578-0600 AMC CALIBRATION STANDARD**
(7-amino-4-methylcoumarin; MW=175)
FORM: 50 µM in 50 mM Glycine, pH 8.7, 1 mM EDTA
STORAGE: -70°C
QUANTITY: 600 µL

**BML-PI142-9090 INHIBITOR** (P32/98; MW=260.4)
FORM: 1 mM in DMSO
STORAGE: -70°C
QUANTITY: 20 µl

**BML-KI576-0020 ASSAY BUFFER**
(50 mM Glycine, pH 8.7, 1 mM EDTA)
FORM: Liquid in screw-cap plastic bottle
STORAGE: -20 or -70°C
QUANTITY: 20 mL
80-2407 ½-VOLUME CLEAR & ½-VOLUME NBS WHITE MICROPLATE – 1 EACH

STORAGE: Room temperature.

OTHER MATERIALS NEEDED

- Microplate reader capable of measuring $A_{405}$ to $\geq3$-decimal accuracy, or fluorescence at wavelengths of approximately 380 nm (excitation)/ 460 nm (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
- Biological samples to be tested
IMPORTANT NOTES

Note on kit storage: Store all kit components except the microplates (room temperature) at -70°C for the highest stability. The DPPIV enzyme, meant for use as positive control, 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. Do not maintain diluted components at reaction temperature (e.g. room temperature) for an extended period of time prior to running the assay.

Note on assay design: Either colorimetric (using BML-P188-9091 substrate) or fluorometric (using BML-P189-9091) format can be used. One format may work better than the other depending on the type and composition of biological samples used, but in general, either is suitable. Some biological samples, such as saliva and urine, exhibit lower inherent DPPIV activity. With such samples, it is recommended they be concentrated, a higher GP-pNA substrate concentration be used (as high as 4 mM has been used), or the more-sensitive fluorogenic substrate (GP-AMC) be used (see Figure 2). The suggested experimental method outlined below is one way to design the assay (reaction velocity over time; comparing relative reaction velocities among samples; etc.). Depending on the experimental objective, you may wish to design your experiment in a different manner. Alternate experimental design suggestions (stopped reactions; using chromophore/fluorophore standard curves to determine amount substrate hydrolyzed; etc.) are outlined in the sections entitled “Alternative Experimental Setups” and “Alternative Data Analyses”, below. Because DPPIV retains more activity than DPP8 and DPP9 when the assay temperature is dropped to room temperature (see Background, above, and Figure 5), it is suggested that assays be run at room temperature.

Collection, processing, and storage of biological samples: In general, samples should be collected on ice, processed and frozen as soon as possible, and stored in aliquots at -20 or -80°C, although Hama et al. reported that urinary DPPIV activity is stable at 4 °C for a week. Serum can be clotted at room temperature for one hour, but should be processed as soon as possible. With regard to plasma: EDTA, citrate, and heparin plasma have been used in this assay (see Figures 1-5; also refs 11,14-22,37). Samples should be clarified by centrifugation (700-1300xg to separate plasma and serum, then >10,000 x g to clarify; other sample types >10,000 x g) or filtration (0.2 µM; in particular saliva, to remove bacteria).
SUGGESTED EXPERIMENTAL DESIGN

To start assay:
1. Defrost kit components and samples, 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, or biological sample, is thawed.
2. Dilute inhibitor (P32/98) 1/10 in assay buffer. Example: Add 3 µL inhibitor to 27 µL assay buffer, in a separate tube.
3. For colorimetric assay (at A\textsubscript{405nm}, using clear microplate): Dilute substrate (H-Gly-Pro-pNA) 1/20 in assay buffer (10 µL is needed per well). Example: Add 15 µL substrate to 285 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) 1/25 in assay buffer (10 µL is needed per well). Example: Add 12 µL substrate to 288 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. room temperature).
7. After a brief thawing, quickly centrifuge the vial of positive control DPPIV (BML-SE434-9091) to bring contents to bottom of tube. Dilute 1/25 in assay buffer to produce required quantity (20 µL is needed per well). For example, dilute 2 µL DPPIV into 48 µL assay buffer.
8. Add 20 µL DPPIV to the appropriate wells. Final amount of DPPIV will be 0.26 mU per well.
9. Add 20 µL biological sample to the appropriate wells. Two or three different dilutions of each biological sample may be desired (see reference 16 and Figure 3).
10. Add 10 µL P32/98 inhibitor (diluted in step 2) to the “Inhibitor” wells. Final inhibitor concentration is 10 µM.
11. Incubate plate for 10-30 min at assay temperature to allow inhibitor/enzyme interaction.
12. Start assay by the addition of 10 µL H-Gly-Pro-pNA substrate (diluted in step 3) OR 10 µL H-Gly-Pro-AMC substrate (diluted in step 4), which have been equilibrated to assay temperature (e.g. room temperature). Enough of each substrate is provided to perform at least 96 assays at 500 µM for the chromogenic substrate or 200 µM for the fluorogenic substrate. If higher...
concentrations are used, these substrates are available separately (BML-P188 and BML-P189) from Enzo Life Sciences.

13. Read plate continuously, at A₄₀₅nm 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**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Assay Buffer (µL)</th>
<th>Biological Sample (µL)</th>
<th>DPPIV (13 µU/µL)</th>
<th>Control Inhibitor‡ (µL)</th>
<th>Substrate (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank 1</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Blank 2 Ω</td>
<td>80</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DPPIV Control 1</td>
<td>70</td>
<td>0</td>
<td>20 µL</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>DPPIV Control 2§</td>
<td>50</td>
<td>20</td>
<td>20 µL</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>DPPIV + Inhibitor</td>
<td>60</td>
<td>0</td>
<td>20 µL</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sample*</td>
<td>70</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Sample + Inhibitor</td>
<td>60</td>
<td>20</td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

**All should be performed in duplicate.**

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

Ω Controls for any optical effects exerted by the biological sample.

§ This is an optional control that looks for effects of the biological sample on DPPIV activity.

* "Sample" refers to biological test sample.
Figure 1. Twenty microliters of undiluted plasma or serum were assayed at 25°C (30 minute read at 405 nm) with GP-pNA (500 µM). Negative control contained substrate in assay buffer.

Figure 2A. Twenty microliters of biological sample were assayed at 25°C (30 minute read at 380/460nm) with GP-AMC (200 µM). Negative control contained substrate in assay buffer. Possible GP-AMC substrate depletion by undiluted plasma and serum became apparent after approximately 13 minutes. Unconcentrated urine and saliva activity levels were 40 and 80 times lower than plasma or serum, but readily detectable (see Figure 2B).
**Figure 2B.** Twenty microliters of unconcentrated urine or saliva were assayed at 25°C (30 minute read) with GP-AMC (200 µM).

**Figure 3.** Twenty microliters of different dilutions (in assay buffer) of heparin plasma and serum were added to the assay (read at 25°C for 20 minutes) using 200 µM GP-AMC substrate. The graph shows percent activity relative to 20 µL undiluted plasma/serum. A 1:10 dilution shows more apparent activity than undiluted, indicating interference by more concentrated serum or plasma samples. Note: a 1:500 dilution of serum was not tested.
**Figure 4.** Plasma or serum (20 µL undiluted) was incubated with different concentrations P32/98 DPPIV inhibitor for 20 minutes at 25°C, then assayed with 200 µM GP-AMC (20 minute read).

**Figure 5.** Plasma or serum (20 µL diluted 1:10 with assay buffer) was incubated for 20 minutes at 25°C or 37°C with added DPPIV enzyme and/or P32/98 inhibitor (10 µM), then assayed at the same temperature with 500 µM GP-pNA substrate (20 minute read). Temperature has little or no effect on reaction velocity nor inhibition of plasma and serum activity. Negative controls contained no biological sample.
DATA ANALYSIS

1. Plot data as $A_{405\text{nm}}$ or Relative Fluorescence Units (RFU) versus time for each sample (see Figures 1 and 2).
2. Determine the time range over which the reaction is linear (see Figure 2A).
3. Obtain a “best fit” line for the data points and determine the slope.
4. Average the slopes of duplicate samples.
5. If the blanks have significant slopes, subtract these numbers from the slopes for all samples.
6. The supplied inhibitor, P32/98, is specific for DPPIV and can be used to reveal substrate-hydrolyzing activity not contributed by DPPIV.
7. To compare relative activities among biological samples:
   For example, in Figure 2B, the average slope of the negative control was 0.074 RFU/sec; the average slope of urine was 1.14 RFU/sec; the average slope of saliva was 0.537 RFU/sec. Thus, the activity in urine was \([(1.14-0.074)/(0.537-0.074)]\) 2.3 times that in saliva.

ALTERNATIVE EXPERIMENTAL SETUPS

1. Instead of a continuous (kinetic) assay, (e.g., reading every minute for 30 minutes) one can also perform a
   a) zero-minute read and a final read, as in references 14, 17, 22.
   b) Stopped reaction using acetic acid, as in references 16, 20, 23, 27, 34, 40.
   Note that in these one-dimensional endpoint assays, it is particularly important that the optimal amount of sample and incubation time be determined empirically. Ferraris et al.16 compared three different dilutions of each biological sample in their stopped reactions in order to ensure linearity (see Figure 3 for an illustration of dilution effects).
2. Instead of comparing relative activities among samples, one can
   a) Compare activities to a DPPIV standard curve, as in reference 26. For µg/µL concentration of DPPIV in this kit, contact Enzo Technical Services with the kit catalog (BML-AK498) and lot numbers.
   b) Build a standard curve of pNA (when using the chromogenic substrate) or AMC (when using the fluorogenic substrate) in order to determine the activity of the samples in pmol substrate hydrolyzed/min. See the section below, “Alternative Data Analyses”.
3. Samples can be normalized in various ways. For example, by protein content (as in reference 34 for saliva and references 16 and 21 for plasma) or creatinine (as in reference 17 for tissue and 23 and 25 for urine).

4. If ex vivo inhibitor assays are being performed, as noted by Edmondson et al.12, since the biological sample containing the in-vivo-delivered inhibitor is diluted in the assay, this may change the kinetics of interaction between DPPIV in the biological sample and the inhibitor, if the inhibitor is reversible.
Product Manual

ALTERNATIVE DATA ANALYSIS

1. To find the activity of the samples expressed as pmol substrate/min using chromogenic substrate:
   Determine microplate reader conversion factor:
   a) Add 100 µL calibration standard (BML-KI577, p-nitroaniline; 80 µM concentration) to 2 wells of the clear microplate. In Tris buffer the extinction coefficient for p-nitroaniline at 405 nm is ~9700 M⁻¹cm⁻¹ (reference 44).
   b) Determine the average A₄₀₅nm using 100 µL assay buffer as a blank.
   c) Calculate the conversion factor.

   Conversion factor (µM/OD) = 80/average A₄₀₅ from step b

d) Alternatively, build a standard curve and use the slope (µM/OD) as the conversion factor. For example, Dimitrijević et al.¹⁴ used 0-80 µM pNA.
e) If there is any optical effect exerted by the biological sample (revealed by Blank 2 in Table 1), the conversion factor should be determined in the presence of biological sample.
f) 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 = 80 µM/0.80 OD = 100 µM/OD

Activity of a control sample =
4.04E-03 (OD/min) x 100(µM/OD) x 100(µL) = 40.4 pmol/min GP-pNA

2. To find the activity of the samples expressed as pmol substrate/min using fluorogenic substrate:
a) Determine fluorimeter reader conversion factor for the 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 (BML-KI578), as provided (50 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. For example, Kirino et al.\textsuperscript{17} used 0-50 µM. The slope of the standard curve can then be used as the µM/RFU conversion factor.

b) If there is any optical effect exerted by the biological sample (revealed by Blank 2 in Table 1), the conversion factor should be determined in the presence of biological sample.

c) Calculate the activity as pmol/min:

\[
\text{Activity (pmol/min)} = \text{slope (RFU/sec)} \times 60 \text{sec/min} \times \text{conversion factor (µM/RFU)} \times \text{assay vol (µL)}
\]
REFERENCES

32. Two highly conserved glutamic acid residues in the predicted ß propeller domain of dipeptidyl peptidase IV are required for its enzyme activity: C.A. Abbott et al.; FEBS Lett. 458, 278 (1999)