Background

Dipeptidyl peptidase IV (DPP (IV)), is also known as CD26 (cluster of differentiation 26 or T-cell activation antigen CD26), or is adenosine deaminase complexing protein 2. It is a multifunctional membrane-bound glycoprotein present of the surface of most cell types and is associated with immune regulation, signal transduction, and apoptosis. [1] In humans, DPP (IV) is ubiquitously expressed in almost all organs and tissues, with the highest expression in kidney, small intestine, and placenta.[2] A soluble form of DPP (IV) can be found in human serum and seminal fluid and has been characterized as a proteolytic derivative of the membrane-bound form.[3] DPP (IV) is a serine exopeptidase that cleaves X-proline or X-alanine dipeptides from the N-terminus of polypeptides. There are at least 63 substrates which can bind specifically to DPP (IV) including growth factors, chemokines, neuropeptides, and vasoactive peptides.[4]

DPP (IV) inhibitors have emerged as a new class of oral antidiabetic agents.[5,6] These inhibitors promote glucose homeostasis by inhibiting DPP (IV), the enzyme responsible for degrading two key glucoregulatory hormones: glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). GLP-1 extends the action of insulin while suppressing the release of glucagon. Clinical studies have evaluated the potential for DPP (IV) inhibition to reduce glucagon levels, delay gastric emptying, and stimulate insulin release. DPP (IV) inhibitors appear to have excellent therapeutic potential in the management of type 2 diabetes. DPP (IV) also plays an important role in tumor biology, and is useful as a marker for various cancers, with its levels either on the cell surface or in the serum increased in some neoplasms and decreased in others.[7,8]

About This Assay

DPP (IV) Inhibitor Screening Assay Kit provides a convenient fluorescence-based method for screening DPP (IV) inhibitors. The assay uses the fluorogenic substrate, Gly-Pro-Aminomethylcoumarin (AMC), to measure DPP (IV) activity. Cleavage of the peptide bond by DPP releases the free AMC group, resulting in fluorescence that can be analyzed using an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm.

Material Supplied

Kit will arrive packaged as a -80℃ kit. For best results, remove components and store as stated below.

Item	Quantity	Storage
DPP Assay Buffer (10X)	1 vial	-20℃
DPP IV (human recombinant)	2 vials	-80℃
DPP Substrate	1 vial	-20℃
Half Volume 96-Well Plate (white)	1 plate	Room temperature
96-Well Cover Sheets	1 cover	Room temperature

WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Materials Needed But Not Supplied

- ✓ A fluorometer with the capacity to measure fluorescence using an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm
- ✓ Adjustable pipettes and a repeat pipettor
- ✓ A source of pure water; glass distilled water or HPLC-grade water is acceptable

Storage and Stability

This kit will perform as specified if stored as specified in the **Materials Supplied** section, and used before the expiration date indicated on the outside of the box.

Reagent Preparation

- ✓ DPP Assay Buffer (10X) - The vial contains 5 ml of buffer. Dilute 3 ml of Assay Buffer concentrate with ml of HPLC-grade water. This final Buffer (20 mM Tris-HCl, pH 8.0, containing mM NaCl, and 1 mM EDTA) should be used in the assay and for diluting reagents. When stored at -20℃, this diluted buffer is stable for at least six months.
- ✓ DPP IV (human recombinant) - Each vial contains 120 µl of human recombinant DPP (IV). Thaw the enzyme on ice, add 480 µl of diluted Assay Buffer to the vial, and vortex. The diluted enzyme is stable for two hours on ice. One vial of enzyme is enough enzyme to assay 60 wells. Use the additional vial if assaying the entire plate.
- ✓ DPP Substrate - The vial contains 300 µl of 5 mM H-Gly-Pro conjugated to aminomethylcoumarin (AMC). Dilute 120 µl with 2.88 ml of diluted Assay Buffer and vortex. This will be enough substrate solution for 60 wells. Prepare additional substrate as needed. The substrate solution is stable for six hours at room temperature. The addition of 50 µl to the assay yields a final concentration of 100 µM substrate.
  NOTE: The K_m value for the peptide is 17.4 µM. The substrate concentration in the assay may be reduced by dilution with assay buffer at the user's discretion, particularly when assaying for competitive inhibitors.

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% initial activity and three wells designated as background wells. We suggest that each inhibitor sample be assayed in triplicate and that you record the contents of each well. A typical layout of samples and compounds to be measured in triplicate is given in Figure 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	A	A	A	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

BW - Background Wells
A - 100% Initial Activity Wells
1-30 - Inhibitor Wells

Figure 1. Sample plate format

Pipetting Hints

- ✓ It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps to maintain more precise incubation times.
- ✓ Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- ✓ Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- ✓ The final volume of the assay is 100 µl in all the wells.
- ✓ All reagents except the enzyme must be equilibrated to room temperature before beginning the assay.
- ✓ It is not necessary to use all the wells on the plate at one time.
- ✓ We recommend assaying samples in triplicate, but it is the user's discretion to do so.
- ✓ The assay is performed at 37℃.
- ✓ If the appropriate inhibitor concentration is not known, it may be necessary to assay at several dilutions. A dilution series of each inhibitor can be performed to determine IC_{50} values.

- ✓ Thirty inhibitor samples can be assayed in triplicate or forty-five in duplicate.
- ✓ Monitor the fluorescence with an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm

Performing the Assay

1. 100% Initial Activity Wells - add 30 µl of assay buffer, 10 µl of diluted DPP, and 10 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
2. Background Wells - add 40 µl of assay buffer and 10 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
3. Inhibitor Wells - add 30 µl of assay buffer, 10 µl of diluted DPP, and 10 µl of inhibitor* to three wells.
4. Initiate the reactions by adding 50 µl of diluted substrate solution to all the wells being used.
5. Cover the plate with the plate cover and incubate for 30 minutes at 37℃.
6. Remove the plate cover and read the fluorescence using an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples.

Inhibitors can be dissolved in assay buffer or dimethylsulfoxide and should be added to the assay in a final volume of 10 µl. Ethanol and methanol dramatically reduce enzyme activity and thus they are not recommended for dissolving inhibitors. In the event that the appropriate concentration of inhibitor needed for DPP inhibition is completely unknown, we recommend that several concentrations of the compound be assayed.

Well	Assay Buffer	DPP	Solvent	Inhibitor	Substrate Solution
100% Initial Activity	30 µl	10 µl	10 µl	-	50 µl
Background	40 µl	-	10 µl	-	50 µl
Inhibitor	30 µl	10 µl	-	10 µl	50 µl

Table 1. Pipetting summary

Calculations

1. Determine the average fluorescence of 100% Initial Activity, Background, and inhibitor wells.
2. Subtract the fluorescence of the background wells from the fluorescence of the 100% initial activity and inhibitor wells.
3. Determine the percent inhibition for each compound. To do this, subtract each inhibitor sample value from the 100% initial activity sample value. Divide the result by the 100% initial activity value and then multiply by 100 to give the percent inhibition.

$$\% \text{ Inhibition} = \left[\frac{\text{Initial Activity - Inhibitor}}{\text{Initial Activity}} \right] \text{ x } 100$$

4. If multiple concentrations of inhibitor are tested, graph either the Percent Inhibition or Percent Initial Activity as a function of the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition). An example of DPP (IV) inhibition by Ile-Pro-Ile (Diprotin A) is shown in Figure 2 .

Precision:

When a series of sixteen DPP measurements were performed on the same day, the intra-assay coefficient of variation was 3.9%. When a series of sixteen DPP measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 4.1%.

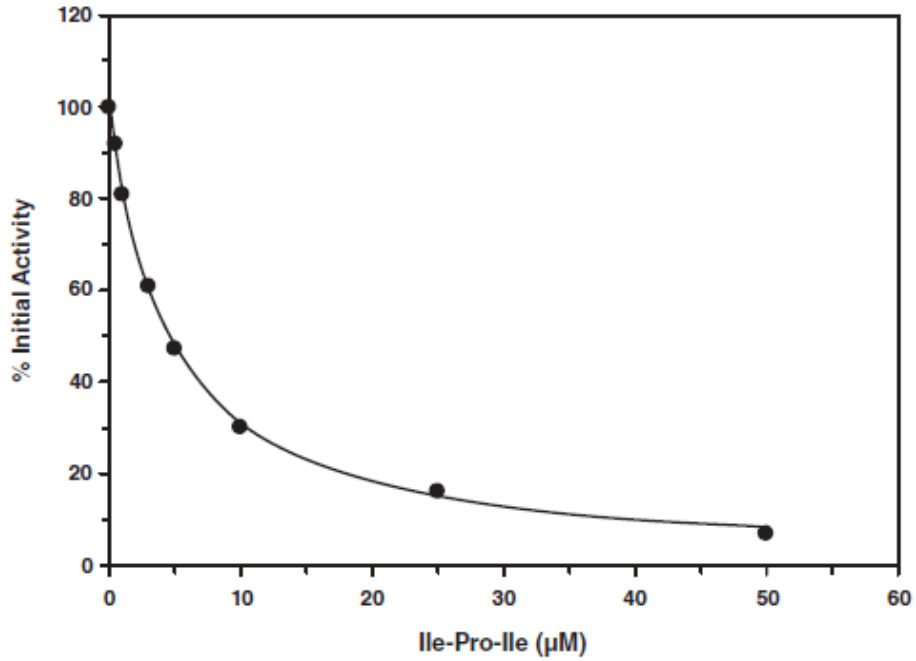

Figure 2. Inhibition of DPP (IV) by Ile-Pro-Ile (IC_{50} = 4.5 µM)

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Carefully tap the side of the plate with your finger to remove bubbles B. Be careful not to splash the contents of the wells
No fluorescence above background is seen in the Inhibitor wells	Inhibitor concentration is too high and inhibited all of the enzyme activity	Reduce the concentration of the inhibitor and re-assay
The fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read
No inhibition was seen with the inhibitor	A. The inhibitor concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the inhibitor concentration and re-assay

References

1. Morimoto, C., Lord, C.I., Zhang, C., *et al.* Role of CD26/dipeptidyl peptidase IV in human immunodeficiency virus type 1 infection and apoptosis. *Proc. Nat Acad. Sci. USA* **91**, 9960-9964 (1994).
2. Fleischer, B. CD26: A surface protease involved in T-cell activation. *Immunology Today* **15(4)**, 180-184 (1994).
3. Heike, M., Möbius, U., Knuth, A., *et al.* Tissue distribution of the T cell activation antigen Ta1. Serological, immunohistochemical and biochemical investigations. *Clin. Exp. Immunol.* **74**, 431-434 (1988).
4. Durinx, C., Lambeir, A.-M., Bosmans, E., *et al.* Molecular characterization of dipeptidyl peptidase activity in serum. Soluble CD26/dipeptidyl peptidase IV is responsible for the release of X-Pro dipeptides. *Eur. J. Biochem* **267**, 5608-5613 (2000).
5. Lambeir, A.-M., Pereira, J.F.D., Chacón, P. , *et al.* A prediction of DPP IV/CD26 domain structure from a physico-chemical investigation of dipeptidyl peptidase IV (CD26) from human seminal plasma. *Biochim. Biophys. Acta* **1340**, 215-226 (1997).
6. Mentlein, R. Dipeptidyl-peptidase IV (CD26)-role in the inactivation of regulatory peptides. *Regul. Pept.* **85**, 9-24 (1999).
7. Langley, A.K., Suffoletta, T.J., and Jennings, H.R. Dipeptidyl peptidase IV inhibitors and the incretin system in type 2 Diabetes Mellitus. *Pharmacotherapy* **27**, 1163-1180 (2007).
8. Doupis, J. and Veves, A. DPP4 inhibitors: A new approach in diabetes treatment. *Advances in Therapy* **25(7)**, 627-643 (2008).
9. Kikkawa, F., Kajiyama, H., Shibata, K., *et al.* Dipeptidyl peptidase IV in tumor progression. *Biochim. Biophys. Acta* **1751**, 45-51 (2005).

10. Havre, P.A., Abe, M., Urasaki, Y., *et al.* The role of CD26/dipeptidyl peptidase IV in cancer. *Frontiers in Bioscience* **13**, 1634-1645 (2008).