

ASSAY FOR DPPIV ACTIVITY USING A HOMOGENEOUS, LUMINESCENT METHOD

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The DPPIV-Glo™ Protease Assay is a homogeneous, luminescent assay that measures activity of DPPIV, a serine protease that is involved in a variety of biological processes including immune regulation, signal transduction and apoptosis. In the assay, adding a single DPPIV-Glo™ Reagent results in DPPIV cleavage of the supplied substrate and generation of a "glow-type" luminescent signal that is proportional to DPPIV activity. The assay is easily amenable to high-throughput screening applications.

Introduction

Dipeptidyl peptidase IV (DPPIV) is a serine protease that cleaves N-terminal dipeptides from polypeptides with L-proline or L-alanine at the penultimate position (1). DPPIV is a multifunctional protein expressed on the surface of several cell types including epithelial, endothelial, and lymphoid cells. It is identical to the T cell activation antigen CD26 and the adenosine deaminase binding protein (2), and it is also released as a soluble form in plasma (3). The substrates of CD26/DPPIV include a wide variety of proline-containing peptides such as growth factors, chemokines, neuropeptides, and vasoactive peptides. DPPIV is involved in immune regulation, signal transduction, and apoptosis, and appears to play an important role in tumor progression (4). Importantly, DPPIV is a therapeutic target for type II diabetes due to its role as a serum protease that cleaves incretin hormones of the glucagon family of peptides and thus regulates glucose homeostasis (3,5,6). Studies indicate that a DPPIV inhibitor improves impaired glucose tolerance (1,7).

The DPPIV-Glo™ Assay demonstrates improved sensitivity compared to fluorescence-based DPPIV assays, allowing the researcher to use less enzyme while still achieving appropriate Z' values.

DPPIV-Glo™ Protease Assay Principle

The DPPIV-Glo™ Protease Assay^(a,b) is a homogeneous, luminescent assay that measures DPPIV activity. The assay provides a proluminescent DPPIV substrate, Gly-Pro-aminoluciferin, which is added to a buffer system optimized for DPPIV and luciferase activities. In this homogeneous, coupled-enzyme format, adding a single DPPIV-Glo™ Reagent results in DPPIV cleavage of the substrate and generation of a "glow-type" luminescent signal produced by the luciferase reaction (Figure 1). The DPPIV-Glo™ Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) that is formulated to generate a stable "glow-type" luminescent signal across a wide range of assay conditions.

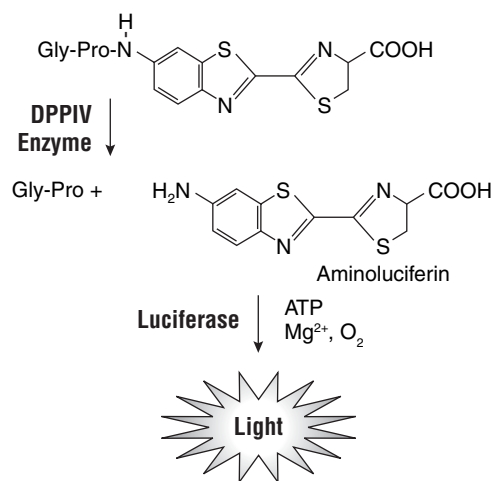


Figure 1. The proluminescent substrate containing the Gly-Pro sequence recognized by DPPIV. Following DPPIV cleavage, the substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and the production of light.

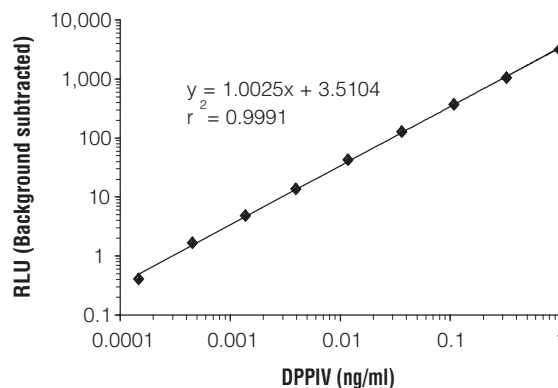


Figure 2. Titration of DPPIV enzyme assayed in 96-well plates using the DPPIV-Glo™ Assay. Recombinant human DPPIV was serially diluted in Tris buffer (10mM Tris-HCl (pH 8.0) + 0.1% Prionex® as a carrier). Luminescence was recorded as relative light units (RLU) on a Dynex MLX® luminometer 30 minutes after adding the DPPIV-Glo™ Reagent. The assay is linear over at least 3 logs of DPPIV concentration ($r^2 = 0.9991$, slope = 1.0025). Each point represents the average of 4 wells. The no-DPPIV control value was subtracted from each. (Average background RLU = 11.4). The r^2 and slope values were calculated after transforming the data to a \log_{10} - \log_{10} plot.

Luminescent DPPIV Protease Assay

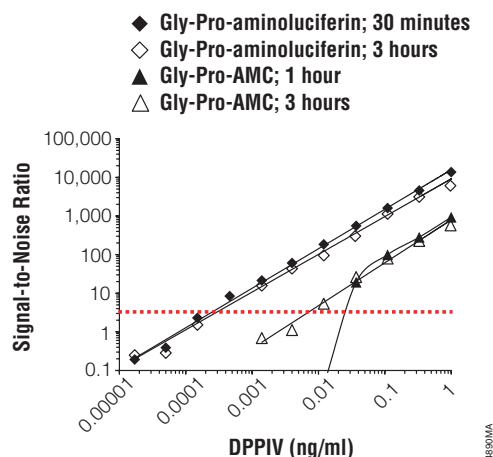


Figure 3. Sensitivity of the DPPIV-Glo™ Protease Assay compared to a fluorescent assay. Recombinant DPPIV enzyme was titrated and assayed in 96-well plates using the DPPIV-Glo™ Assay or a Gly-Pro-AMC fluorescent substrate. Luminescence and fluorescence were monitored at various times on a Dynex MLX® luminometer and a Labsystems Fluoroscan Ascent fluorometer, respectively. The results are plotted as signal-to-noise ratios. The limit of detection is defined as the amount of DPPIV giving a signal-to-noise ratio >3 (dashed line). The bioluminescent assay demonstrated a limit of detection of <0.3pg/ml in 30 minutes, whereas the fluorescent assay demonstrated a limit of detection of ~6pg/ml after 3 hours.

The "add-mix-measure" format of the DPPIV-Glo™ Protease Assay is designed for use with multiwell plate formats, making it ideal for automated high-throughput screening (HTS). This coupled-enzyme system, with simultaneous DPPIV cleavage of substrate and luciferase consumption of the released aminoluciferin, results in a luminescent signal that is proportional to the amount of DPPIV activity present. The assay has

a broad dynamic range of DPPIV concentration, resulting in exceptional sensitivity (Figure 2).

More Sensitive than Comparable Fluorescent Assay

We compared the luminescent DPPIV-Glo™ Protease Assay to a fluorescent DPPIV assay that uses the comparable Gly-Pro-AMC substrate. The DPPIV-Glo™ Protease Assay is significantly more sensitive at all time points (Figure 3). Signal-to-noise ratios [(mean signal – mean background) / standard deviation of the background] were used to compare the two formats. The signal-to-noise ratio is tenfold better for the DPPIV-Glo™ Protease Assay, and the limit of detection is at least tenfold lower than the fluorescent assay (Figure 3). Although the sensitivity of the fluorescent assay improves over time due to the accumulation of released free AMC, even after 3 hours, the signal-to-noise ratios are still much lower than those for the luminescent DPPIV-Glo™ Protease Assay. The sensitivity of the homogeneous DPPIV-Glo™ Protease Assay remains relatively constant over extended periods due to the coupled-enzyme format. The DPPIV and luciferase enzyme activities rapidly reach a steady-state so that the luminescent signal peaks in 20–30 minutes and is then maintained for several hours with minimal loss. The half-life for the assay is greater than 4 hours (Figure 4). This provides a rapid, sensitive, and flexible DPPIV activity assay.

Ideal for High-Throughput DPPIV Inhibitor Screens

The sensitivity and speed of the assay and the flexibility in optimum read time makes the DPPIV-Glo™ Assay a useful tool for inhibitor screening. Since compound libraries are frequently stored in DMSO, we determined the effect of DMSO on the assay. Concentrations up to 5% DMSO showed

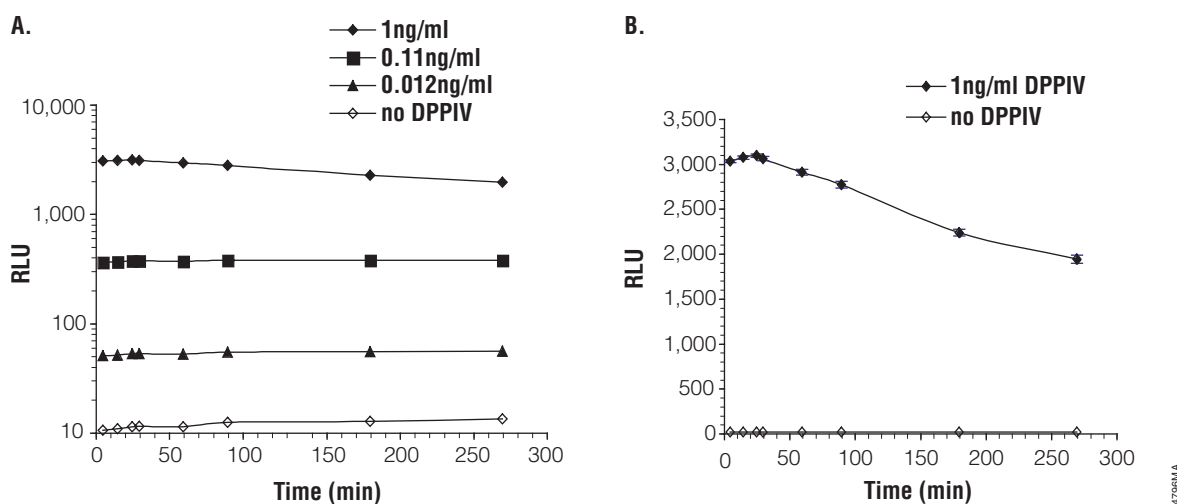


Figure 4. Signal stability of the DPPIV-Glo™ Protease Assay. Recombinant DPPIV enzyme was titrated and assayed in 96-well plates using the DPPIV-Glo™ Assay. Luminescence was monitored for 4.5 hours on a Dynex MLX® luminometer. **Panel A.** Over a broad range of DPPIV concentration, the assay gives a stable signal for several hours, as shown on a log scale. **Panel B.** The luminescent signal has a half-life greater than 4 hours, shown here for the 1ng/ml DPPIV concentration on a linear scale. All points represent the average of 4 wells.

Luminescent DPPIV Protease Assay

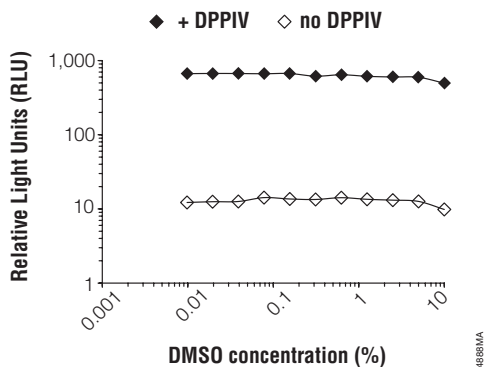
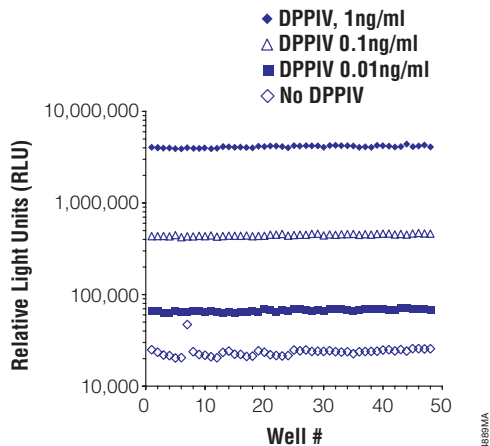


Figure 5. Effect of DMSO on the DPPIV-Glo™ Assay. Various concentrations of DMSO were combined with purified DPPIV enzyme (0.25ng/ml) or a buffer control and assayed in 96-well plates using the DPPIV-Glo™ Assay. Luminescence was monitored after 30 minutes on a Dynex MLX® luminometer. All points represent the average of 4 wells.



DPPIV Concentration	Z'-value
1.0ng/ml	0.91
0.1ng/ml	0.89
0.01ng/ml	0.59

Figure 7. Determination of Z' values. Recombinant DPPIV was diluted in buffer and added to wells at concentrations of 1.0, 0.1, and 0.01ng/ml. Half of a 96-well plate (48 wells) was used for each concentration and for the buffer control. Luminescence was recorded 30 minutes after adding the DPPIV-Glo™ Reagent on a Turner Veritas™ Microplate Luminometer (Note: The Veritas™ Microplate Luminometer (Cat.# E6501) gives higher RLUs than the Dynex MLX®). The table denotes the Z' values calculated for each dose as per Zhang *et al.* (10).

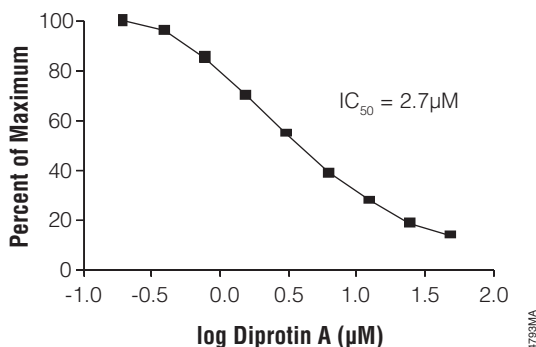


Figure 6. Determination of IC₅₀ values. The inhibitor concentration that results in 50% inhibition (IC₅₀) was determined for the DPPIV competitive inhibitor, Diprotin A, using the DPPIV-Glo™ Protease Assay. The inhibitor was resuspended in DMSO and serially diluted in 10mM Tris-HCl (pH 8.0) + 0.1% Prionex® and combined with 50pg/ml of DPPIV in 96-well plates. The maximum DMSO concentration is 0.5%. Luminescence was recorded 30 minutes after reagent addition, and GraphPad Prism® software was used to calculate the IC₅₀ of 2.7µM.

no effect on the assay. At 10% DMSO, the signal and the background decreased slightly (Figure 5).

We used the DPPIV-Glo™ Assay to test a known DPPIV inhibitor and calculate an IC₅₀. Diprotin A (Ile-Pro-Ile) is a competitive DPPIV inhibitor (8). The inhibitor was resuspended in DMSO and serially diluted in buffer. The maximum DMSO concentration was 0.5%. The DPPIV substrate was used at 0.1µM, a concentration well below the apparent K_m. For competitive inhibitors at a substrate concentration well below K_m, the IC₅₀ = K_i (9). GraphPad Prism® software was used to calculate the IC₅₀ of 2.7µM. This corresponds closely with the published K_i of 3.5µM (8).

In order to assess the quality of the assay, Z' values were calculated in 96-well plates for various concentrations of DPPIV (Figure 7). Values above 0.5 indicate an excellent assay (10). Z' values decrease with decreasing DPPIV concentration, but even at DPPIV concentrations as low as 10pg/ml, the Z' value was 0.59, indicating a robust, suitable assay.

Conclusions

The new DPPIV-Glo™ Protease Assay is the first bioluminescent DPPIV assay. It is a simple, fast and flexible assay that is ideal for high-throughput screening. The assay demonstrates improved sensitivity compared to current fluorescent-based DPPIV assays, allowing the researcher to use less enzyme while still achieving appropriate Z' values. The homogeneous coupled-enzyme format is convenient, requiring only a single reagent addition to the test samples. Maximal sensitivity is achieved in 20–30 minutes, and the signal generated is very stable, having an extended half-life. ■

Luminescent DPPIV Protease Assay

Protocol

DPPIV-Glo™ Protease Assay Technical Bulletin #TB339
(www.promega.com/tbs/tb339/tb339.html)

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Acknowledgments

The authors would like to thank the members of the product launch team for their work on this project: Kay Rashka, B.S., Deborah Bishop, Ph.D., Laurent Bernad, Ph.D., Brian McNamara, Ph.D., Pam Guthmiller, B.S. and Isobel Maciver, Ph.D.

Ordering Information

Product	Size	Cat.#
DPPIV-Glo™ Protease Assay	10ml	G8350
	50ml	G8351
Veritas™ Microplate Luminometer	1 each	E6501

For Laboratory Use

^(a)The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

^(b)U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents pending.

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