# DPPIV-GLO™ PROTEASE ASSAY: A MORE SENSITIVE METHOD FOR MEASURING GLY-PRO CLEAVING ACTIVITY IN SERUM

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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. Here we show that the DPPIV-Glo™ Protease Assay can detect very low levels of Gly-Pro cleaving activity in serum.

## 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 member of the prolyl oligopeptidase (POP) family, a subfamily of serine proteases, which includes DPPIV, prolyl oligopeptidase (POP), DPPII, DPP8, DPP9, and fibroblast activation protein (FAP). Unlike typical serine proteases, the POP family of enzymes is highly selective toward peptides that have a proline residue at the penultimate position (2). 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 (3), and it is often referred to as DPPIV/CD26. DPPIV is also released as a soluble form in plasma (4). It 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, thus regulating glucose homeostasis (4–6). Studies indicate that a DPPIV inhibitor improves impaired glucose tolerance (1,7). Monitoring serum levels of DPPIV activity and the inhibition of that activity will be of increasing importance as several potential DPPIV inhibitors move through clinical trials as therapeutics for type II diabetes (8,9).

We have developed a bioluminescent assay for DPPIV that can detect Gly-Pro cleaving activity in human serum at concentrations as low as 0.0002% serum.

## Titration of DPPIV-Like Activity in Serum

The secreted form of DPPIV/CD26 is reported to be present in very high concentrations in human serum (10). Although other serum proteases may have X-Pro cleaving activity, Durinx *et al.* (10) concluded that 95% of the DPPIV-like activity in serum was attributable to an adenosine deaminase binding protein with characteristics identical to recombinant DPPIV/CD26. They purified 5mg from 18 liters of pooled serum, indicating a serum concentration of approximately

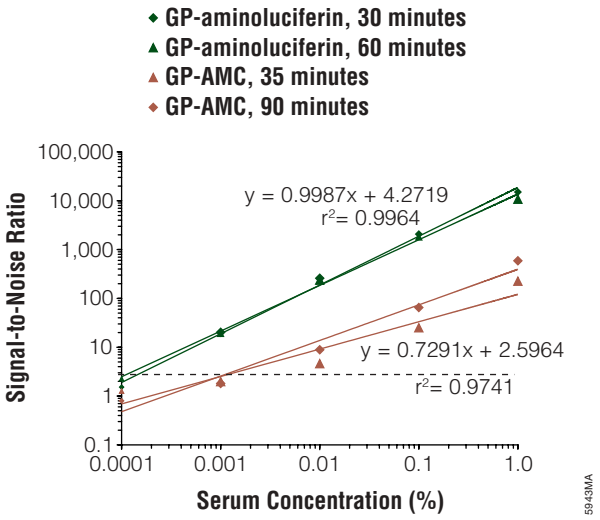


**Figure 1. Human serum titration tested with DPPIV-Glo™ Protease Assay and the fluorescent substrate, GP-AMC.** Human serum was serially diluted in 10mM Tris-HCl (pH 8.0) + 0.1% Prionex® stabilizer. Diluted samples were added to 96-well plates (50µl/well). DPPIV-Glo™ Reagent was added to one plate (50µl/well), and GP-AMC [20µM in 100mM Tris-HCl (pH 8.0)] was added to a second plate. Both substrates were used at a final concentration of 10µM in a final volume of 100µl/well. Luminescence was recorded on a GloMax™ 96 Microplate Luminometer at several time points (30- and 60-minute data shown). Fluorescence was recorded on a LabSystems Fluoroskan Ascent at $355_{Ex}/460_{Em}$. The line equations are for the 30-minute luminescent reading and the 90-minute fluorescent reading. They were derived from the $\log_{10}$-$\log_{10}$ plot of the data. The limit of detection is defined as a signal-to-noise ratio of 3 (dotted line).

0.3µg/ml. The linear range of our DPPIV-Glo™ Protease Assay[a,b] extends from ~0.3pg/ml to 1ng/ml of DPPIV recombinant enzyme (11). Commercially available human serum was tested for DPPIV-like activity (Figure 1). Consistent with levels of DPPIV previously reported in serum (10), the linear range of the bioluminescent assay extended from ~1% to 0.0001% serum, and the limit of detection was 0.0002% serum (Figure 1). The same titration of serum was tested with a comparable fluorescent substrate, GP-AMC (Figure 1). The fluorescent assay lost linearity below 0.01% serum, and the limit of detection was more than tenfold higher than the limit of detection with the DPPIV-Glo™ Assay (Figure 1). The DPPIV-Glo™ Assay reached this sensitivity level in 20 minutes, and the signal-to-noise ratios were consistent

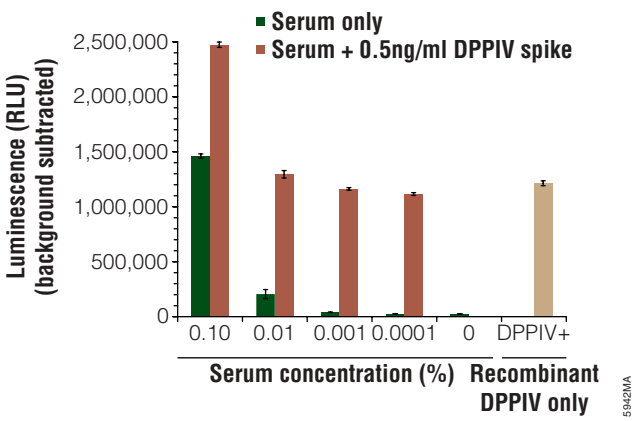


**Figure 2. DPPIV-Glo™ Protease Assay: Human serum titration with recombinant DPPIV added.** Human serum was serially diluted in 10mM Tris-HCl (pH 8.0) + 0.1% Prionex® stabilizer. Diluted samples were added to a 96-well plate, and recombinant DPPIV was added to each to a final concentration of 0.5ng/ml. The DPPIV-Glo™ Reagent was added, and luminescence was recorded after 30 minutes on a GloMax™ 96 Microplate Luminometer. The signal from recombinant DPPIV alone (0.5ng/ml) is shown in yellow.

between 20 and 90 minutes due to the coupled-enzyme, homogeneous format that generates a stable signal (11). The signal-to-noise ratio improved for the fluorescent assay between 35 and 90 minutes, but even after 90 minutes, it still had not reached the sensitivity of the luminescent assay (Figure 1).

## Addition of DPPIV Enzyme to Serum Quantitatively Increases Signal

Spiking serum samples with DPPIV enzyme should increase the DPPIV-Glo™ signal. DPPIV recombinant enzyme (0.5ng/ml) was added into each diluted serum sample from 0.1% to 0.0001% serum. The addition of 0.5ng/ml DPPIV increased the signal at all serum concentrations, and the signals achieved were roughly equivalent to the combined signals of DPPIV alone plus serum alone (Figure 2). The actual signals ranged from 92–95% of the expected combined signal when recombinant DPPIV was added to the serum (Figure 2). When 0.5ng/ml of DPPIV was added to the 0.1% serum sample, the signal increased by 70%. Therefore, if all of the serum activity is attributable to DPPIV, this would indicate that 0.1% serum contains 0.7ng/ml, and undiluted serum would have 0.7µg/ml. This is in the range previously found in pooled human serum (10).

## Inhibition of Serum Gly-Pro Activity with Diprotin A

If the serum DPPIV-like activity is due to DPPIV/CD26, then diprotin A, a selective, reversible inhibitor of DPPIV, should inhibit the activity in serum, and the inhibition should be identical to the inhibition using recombinant DPPIV. The $K_i$ for diprotin A inhibition of DPPIV is reported to be 3.5µM (12). All serum concentrations were tested with the diprotin A

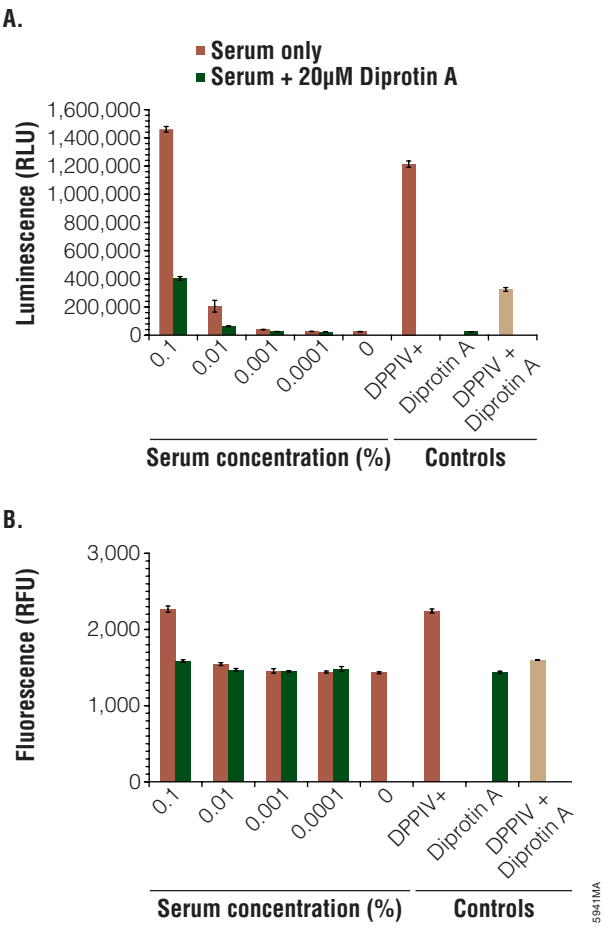


**Figure 3. Inhibition of serum DPPIV-like activity with diprotin A.** Human serum was serially diluted in 10mM Tris-HCl (pH 8.0) + 0.1% Prionex® stabilizer. Diluted samples were added to 96-well plates, and the DPPIV inhibitor, diprotin A, was added to each titration to a final concentration of 20µM. One plate of inhibited and control samples was tested with the DPPIV-Glo™ Protease Assay (**Panel A**), and a duplicate plate was tested with the GP-AMC fluorescent substrate (**Panel B**). Luminescence was recorded after 30 minutes on a GloMax™ 96 Microplate Luminometer. Fluorescence was recorded after 90 minutes on a LabSystems Fluoroskan Ascent at $355_{Ex}/460_{Em}$.

inhibitor at a final concentration of 20µM and then assayed using the luminescent and fluorescent assays (Figure 3). Recombinant DPPIV (0.5ng/ml) was also tested with diprotin A at 20µM as a control. For the DPPIV-Glo™ Assay, recombinant DPPIV activity was inhibited 75% by diprotin A. At 0.1% and 0.01% serum, the luminescent signal was inhibited by 74% and 79%, respectively, consistent with the serum DPPIV-like activity being the same as DPPIV/CD26 (Figure 3, Panel A). These results are also consistent with our $IC_{50}$ curves previously generated for inhibition of DPPIV by diprotin A (11). Because of the elevated background for the fluorescent assay, it was necessary to subtract background in order to determine the inhibition by diprotin A (Figure 3,

Panel B). Using the GP-AMC substrate, the recombinant DPPIV and 0.1% serum were both inhibited by 80%, with 20µM diprotin A, again consistent with serum activity representing DPPIV/CD26 activity. At 0.01% serum and lower, the detection of DPPIV activity is too close to the limit of detection to give accurate inhibition results for the fluorescent assay (Figure 3, Panel B). Consistent with the tenfold improved sensitivity for the luminescent assay (see Figure 1), it is possible to accurately detect inhibition with tenfold less serum (Figure 3, Panel A). The much improved dynamic range of the luminescent assay allows a more accurate determination of inhibition with a broader range of serum dilutions and at lower serum concentrations (compare Figure 3, Panels A and B). Inhibition of human serum with the selective DPPIV inhibitor, diprotin A, was comparable to inhibition of recombinant DPPIV, supporting the previous report indicating that 95% of the DPPIV-like activity in serum derives from the soluble form of DPPIV/CD26 itself (10).

## Summary

We have developed a bioluminescent assay for DPPIV that can detect Gly-Pro cleaving (DPPIV-like) activity in human serum at concentrations as low as 0.0002% serum. Although it is possible that some of the serum DPPIV-like activity is a result of cleaving by other proteases, our results are consistent with a previous study that concludes that 95% of the X-Pro cleaving activity in serum is in fact due to the soluble form of DPPIV/CD26 (10). Recombinant DPPIV could be added to the serum with a quantitative increase in the luminescent signal, and likewise, the activity could be inhibited by the selective DPPIV inhibitor, diprotin A, and that inhibition was comparable to the inhibition of recombinant DPPIV. The quantity of DPPIV-like activity detected in human serum with the DPPIV-Glo™ Protease Assay is also in the range of that reported previously (0.3µg/ml vs. 0.7µg/ml; 10). An extremely sensitive assay allows the user to dilute the serum substantially, which has the advantage of diluting less abundant, non-specific proteases with possible X-Pro cleaving activity. Inhibition can still be monitored accurately even in extremely dilute serum samples using the DPPIV-Glo™ Protease Assay. Finally, the bioluminescent assay is significantly more sensitive than a comparable fluorescent assay, and the dynamic range of the bioluminescent assay is much larger than that of the fluorescent assay. ■

## References


1. Villhauer, E.B. *et al.* (2003) *J. Med. Chem.* **46**, 2774–89.
2. Chien, C-H. *et al.* (2004) *J. Biol. Chem.* **279**, 52338–45.
3. Engel, M. *et al.* (2003) *Proc. Natl. Acad. Sci. USA* **100**, 5063–8.
4. Marguet, D. *et al.* (2000) *Proc. Natl. Acad. Sci. USA* **97**, 6874–9.
5. Zhu, L. *et al.* (2003) *J. Biol. Chem.* **278**, 22418–23.
6. Wiederman, P.E. and Trevillyan, J.M. (2003) *Curr. Opinion Invest. Drugs* **4**, 412–20.
7. Ahren, B. *et al.* (2004) *J. Clin. Endocrinol. Metab.* **89**, 2078–84.
8. Holst, J.J. and Ørskov, C. (2004) *Diabetes* **53,** S197–S204.
9. Mu, J. *et al.* (2006) *Diabetes* **55**, 1695–1704.
10. Durinx, C. *et al.* (2000) *Eur. J. Biochem.* **267**, 5608–13.
11. O'Brien, M.A. *et al.* (2005) *Cell Notes* **11**, 8–11.
12. Leiting, B. *et al.* (2003) *Biochem. J.* **371**, 525–53.


## Protocol

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

## Ordering Information

| Product | Size | Cat.# |
|---|---|---|
| DPPIV-Glo™ Protease Assay* | 10ml | G8350 |
| | 50ml | G8351 |
| GloMax™ 96 Microplate Luminometer | 1 each | E6501 |

*For Laboratory Use.

[a]U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents and patents pending.

[b]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.