



Promega

Technical Bulletin

DPPIV-Glo™ Protease Assay

INSTRUCTIONS FOR USE OF PRODUCTS G8350 AND G8351.

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DPPIV-Glo™ Protease Assay

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1. Description

The DPPIV-Glo™ Protease Assay^(a,b) is a homogeneous, luminescent assay that measures dipeptidyl peptidase IV (DPPIV) activity. 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 both as a soluble form in plasma and on the surface of several cell types including epithelial and endothelial cells and lymphocytes (2). It is identical to the T cell activation antigen CD26 and the adenosine deaminase binding protein (3). DPPIV is a therapeutic target for type II diabetes due to its role in cleaving incretin hormones of the glucagon family of peptides and thus regulating glucose homeostasis (2,4,5).

The DPPIV-Glo™ Protease Assay provides a luminogenic DPPIV substrate, Gly-Pro-aminoluciferin, in a buffer system optimized for DPPIV and luciferase activities. The addition of a single DPPIV-Glo™ Reagent in an “add-mix-measure” format results in DPPIV cleavage of the substrate and generation of a “glow-type” luminescent signal produced by the luciferase reaction (Figure 1). In this homogeneous, coupled-enzyme format, the luminescent signal is proportional to the amount of DPPIV activity present (Figure 2).

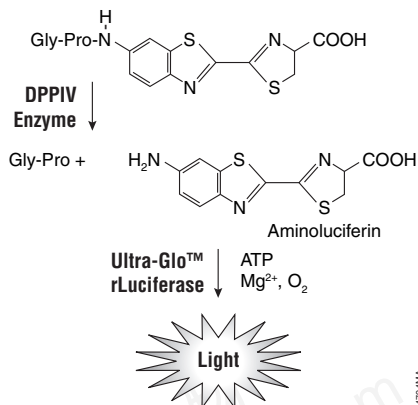


Figure 1. The luminogenic substrate containing the Gly-Pro sequence recognized by DPPIV. Following DPPIV cleavage, the substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and the production of light.

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. The homogeneous DPPIV-Glo™ Protease Assay is designed for use with multiwell plate formats, making it ideal for automated high-throughput screening (HTS) for DPPIV activity. The DPPIV and luciferase enzyme activities reach a steady-state so that the luminescent signal peaks rapidly and is maintained for several hours with minimal loss of signal (Figure 3). This provides for a rapid, sensitive and flexible DPPIV activity assay.

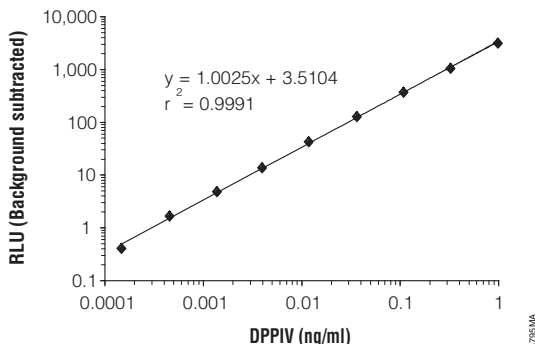


Figure 2. Titration of DPPiV enzyme assayed in 96-well plates using the DPPiV-Glo™ Assay. Recombinant human DPPiV was serially diluted in Tris buffer (10mM Tris-HCl [pH 8.0] + 0.1% Prionex® as a carrier). Luminescence was recorded as relative light units (RLU) on a Dynex MLX® luminometer 30 minutes after adding the DPPiV-Glo™ Reagent. The assay is linear over at least three 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 RLU = 11.4). r^2 and slope were calculated after transforming the data to a \log_{10} - \log_{10} plot.

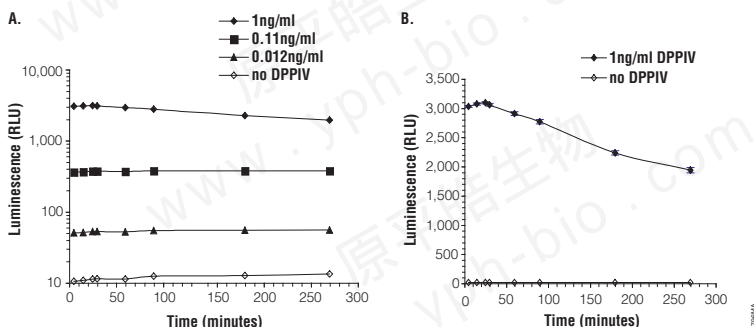


Figure 3. Signal stability of the DPPiV-Glo™ Assay. Purified DPPiV enzyme was titrated and assayed in 96-well plates using the DPPiV-Glo™ Assay. Luminescence was monitored for 4.5 hours on a Dynex MLX® luminometer. **Panel A.** Over a broad range of DPPiV concentration, the assay gives a stable signal 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 four wells.

Assay Advantages

Simplified Method: The homogeneous “add-mix-read” protocol makes the assay highly amenable to automation (Figure 6).

Greater Sensitivity: The assay is more sensitive than fluorescent-based DPPIV assays. In contrast to fluorescent assays, the luminescent assay avoids inherent fluorescent background signals and thus provides excellent signal-to-background readings. The assay is linear over more than three logs of DPPIV concentration and can detect less than 1pg/ml (Figures 2 and 4).

Faster Results: The maximum signal (and maximum sensitivity) of the assay is reached in as little as 30 minutes after reagent addition (Figure 3, Panel B) and, unlike fluorescent assays, is not dependent on accumulation of cleaved product.

More Accurate: The assay provides accurate results for kinetic studies of inhibitors (Figure 5).

More Robust: The assay demonstrates an excellent Z' factor, a statistical value that compares the dynamic range of an assay to data variation in order to assess assay quality. Z' factors greater than 0.5 indicate excellent assay quality (6). The DPPIV-Glo™ Assay gave a Z' factor value of 0.77 in 384-well plates using 1ng/ml of DPPIV.

Amenable to Batch Processing: The coupled-enzyme, homogeneous format results in a continuous signal, providing excellent stability and allowing plates to be read over an extended period of time (Figure 3, Panel A).

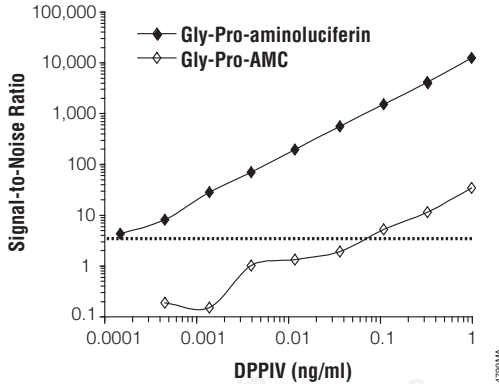


Figure 4. Sensitivity of the DPPiV-Glo™ Protease Assay compared to a fluorescent assay. Recombinant DPPiV enzyme was titrated and assayed in 96-well plates using the DPPiV-Glo™ Assay or a Gly-Pro-AMC fluorescent substrate. Luminescence and fluorescence were monitored after 40 minutes on a BMG FLUOstar combination luminometer/fluorometer. 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 <1pg/ml, whereas the fluorescent assay demonstrated a limit of detection of ~100pg/ml after 40 minutes.

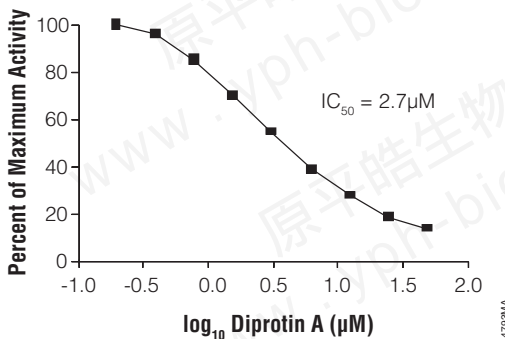


Figure 5. Determination of IC₅₀ values. The inhibitor concentration that results in 50% inhibition (IC₅₀) was determined for the DPPiV competitive inhibitor, Diprotin A (7), using the DPPiV-Glo™ Protease Assay. The inhibitor was resuspended in DMSO and serially diluted in 10mM Tris-HCl (pH 8.0) + 0.1% Prionex® and combined with 50pg/ml of DPPiV in 96-well plates. The maximum DMSO concentration is 0.5%. The DPPiV substrate was used at 0.1μM, a concentration well below the apparent K_m. For competitive inhibitors at a substrate concentration <K_m, the IC₅₀=K_i (8). Luminescence was recorded 30 minutes after reagent addition, and GraphPad Prism® software was used to calculate the IC₅₀ of 2.7μM. This corresponds closely with the published K_i of 3.5μM (7).

2. Product Components and Storage Conditions

Product	Size	Cat.#
DPPIV-Glo™ Protease Assay	10ml	G8350
	50ml	G8351

For Laboratory Use. **Cat.# G8350** provides sufficient reagents for 100 assays at 100µl/assay or 200 assays at 50µl/assay in 96-well plates, or 400 assays at 25µl/assay in 384-well plates. Includes:

- 10ml DPPIV-Glo™ Buffer
- 1 vial DPPIV-Glo™ Substrate
- 1 bottle Luciferin Detection Reagent

Cat.# G8351 provides sufficient reagents for 500 assays at 100µl/assay or 1,000 assays at 50µl/assay in 96-well plates, or 2,000 assays at 25µl/assay in 384-well plates.

Includes:

- 50ml DPPIV-Glo™ Buffer
- 1 vial DPPIV-Glo™ Substrate
- 1 bottle Luciferin Detection Reagent

Storage Conditions: Store the DPPIV-Glo™ Protease Assay components at -20°C protected from light. The DPPIV-Glo™ Buffer may be thawed and stored at 4°C for 2 months with no loss in signal. The DPPIV-Glo™ Substrate resuspended in water can be refrozen and stored at -20°C for 4 weeks with minimal loss of signal. DPPIV-Glo™ Reagent (combined DPPIV-Glo™ Substrate, DPPIV-Glo™ Buffer and Luciferin Detection Reagent) frozen at -20°C for 2 weeks retained approximately 80% of the signal. Avoid exposure to multiple freeze-thaw cycles by storing reconstituted reagents in aliquots at -20°C.

3. Reagent Preparation

Directions are given for performing the DPPIV-Glo™ Protease Assay in a total volume of 100µl using 96-well plates and a luminometer. However, the assay can easily be adapted to different volumes provided that the 1:1 ratio of DPPIV-Glo™ Reagent volume to sample volume is preserved (e.g., 25µl of sample + 25µl DPPIV-Glo™ Reagent in a 384-well format).

Materials to be Supplied by the User

- white multiwell plates
(black plates may be used, but RLUs will be reduced)
- multichannel pipette or automated pipetting station for delivery of DPPIV-Glo™ Reagent
- device (plate shaker) for mixing multiwell plates
- luminometer capable of reading multiwell plates
- DPPIV enzyme (e.g., R&D Systems or Sigma)

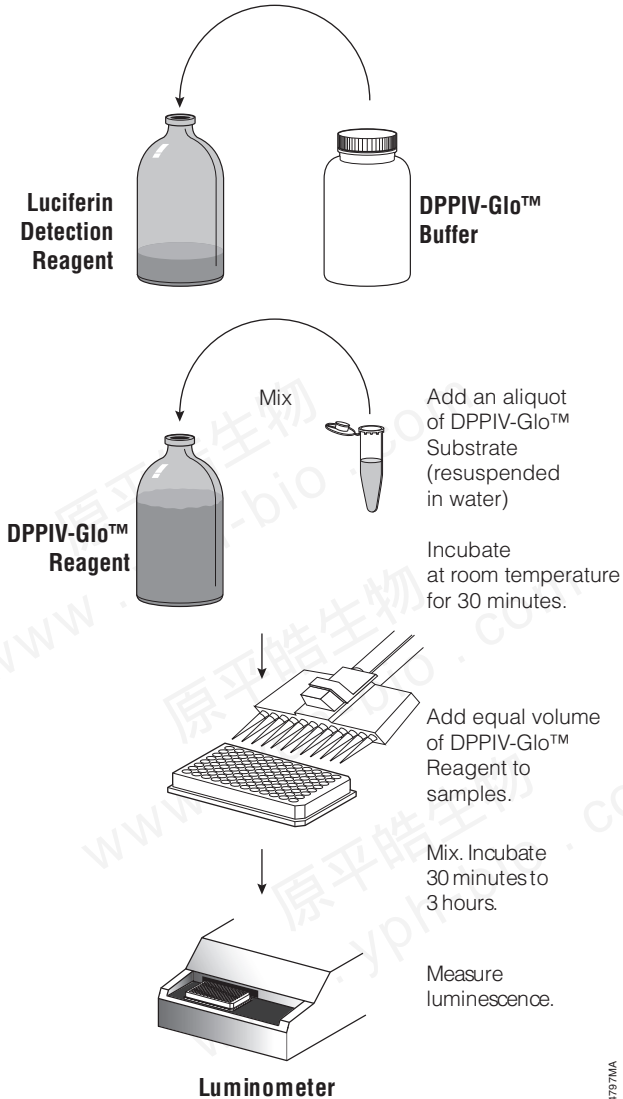


Figure 6. Flow diagram showing preparation and use of the DPPIV-Glo™ Reagent.

DPPIV-Glo™ Reagent Preparation

1. Thaw the DPPIV-Glo™ Buffer and equilibrate to room temperature before use.
2. Equilibrate the lyophilized Luciferin Detection Reagent to room temperature prior to use.
3. Resuspend the DPPIV-Glo™ Substrate by adding ultrapure water (25µl for Cat.# G8350, 110µl for Cat.# G8351) to the substrate vial. Mix by vortexing briefly. This makes a 10mM substrate stock.
4. Reconstitute the Luciferin Detection Reagent in the amber bottle by adding DPPIV-Glo™ Buffer (10ml for Cat.# G8350, 50ml for Cat.# G8351). The Luciferin Detection Reagent should go into solution easily in less than one minute.
5. Prepare the DPPIV-Glo™ Reagent by adding the DPPIV-Glo™ Substrate to the resuspended Luciferin Detection Reagent. For Cat.# G8350, add 20µl of DPPIV-Glo™ Substrate to the 10ml of Luciferin Detection Reagent. For Cat.# G8351, add 100µl of the DPPIV-Glo™ Substrate to the 50ml of Luciferin Detection Reagent. Mix by swirling or inverting the contents to obtain a homogeneous solution. The DPPIV-Glo™ Substrate will be at 20µM concentration in the DPPIV-Glo™ Reagent. The apparent K_m for the substrate is 10µM.

Note: Prepare only enough DPPIV-Glo™ Reagent for your experiment. Any remaining DPPIV-Glo™ Substrate and reconstituted Luciferin Detection Reagent should be stored in aliquots at -20°C.

6. Allow the DPPIV-Glo™ Reagent to sit at room temperature for 30–60 minutes prior to use. This allows the removal of any contaminating free aminoluciferin, ensuring maximal sensitivity. Although free aminoluciferin is not detected by HPLC, it is present in trace amounts (Figure 7).

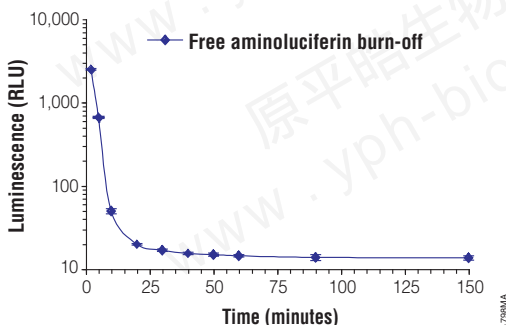


Figure 7. Time course of luminescence of resuspended DPPIV-Glo™ Substrate (Gly-Pro-aminoluciferin) added to the Luciferin Detection Reagent. Trace amounts of free aminoluciferin are present in the substrate and can be “burned-off” by incubation with the Luciferin Detection Reagent. To achieve maximal assay sensitivity with minimal background luminescence, the prepared DPPIV-Glo™ Reagent should be incubated for 30–60 minutes before use. The residual RLU are due to the spontaneous hydrolysis of the substrate.

4. Assay for Detection of DPPIV Activity

4.A. Assay Conditions

Prepare the following reactions to detect DPPIV activity (or inhibition of activity) in purified enzyme preparations:

- **Blank:** DPPIV-Glo™ Reagent + vehicle control for enzyme treatment agent or inhibitor
- **Positive Control:** DPPIV-Glo™ Reagent + vehicle control + purified DPPIV enzyme
- **Assay:** DPPIV-Glo™ Reagent + treatment agent + purified DPPIV enzyme

The blank is used as a measure of any background luminescence associated with the treatment agent vehicle and DPPIV-Glo™ Reagent, and should be subtracted from experimental values. The positive control is used to determine the maximum luminescence obtainable with the purified enzyme system. “Vehicle” refers to the solvent used to dissolve the inhibitor or treatment agent used in the study.

Notes:

1. Prepare the DPPIV-Glo™ Reagent as described in Section 3, and mix thoroughly prior to starting the assay.
2. The final concentration of DPPIV enzyme should be 1ng/ml or less.
3. Use identical enzyme concentrations for the assay and positive control reactions.
4. Gentle mixing may be performed using a plate shaker.
5. The maximal luminescent signal will be reached in ~30 minutes and will be stable for several hours (Figure 3).

4.B. Standard Assay (96-well, 100µl final reaction volume)

1. Add 50µl of DPPIV-Glo™ Reagent to each well of a white or black 96-well plate containing 50µl of blank, control or assay treatment. If reusing tips, be careful not to touch pipet tips to the wells containing samples to avoid cross-contamination. Plates can be covered with a plate sealer if incubating for extended periods (>4 hours).
2. Gently mix the contents of the wells using a plate shaker at 300–500rpm for 30 seconds. Incubate at room temperature for 30 minutes to 3 hours (Figure 3, Panel A). Maximal signal is typically reached within 30 minutes using DPPIV enzyme (Figure 3, Panel B). At this time sensitivity is optimal. Temperature fluctuations will impact the luminescent readings; if the room temperature fluctuates significantly, a constant-temperature incubator may be desired.
3. Record luminescence.

5. General Considerations

Sensitivity

The bioluminescent DPPIV-Glo™ Protease Assay is more sensitive than comparable fluorescent assays for several reasons. Fluorescence substrates generally depend on a shift in the excitation/emission wavelengths after cleavage by the protease; consequently, there is some overlap in the emission spectra of the substrate before and after cleavage, creating substantial inherent background. The luminescent substrate (Gly-Pro-aminoluciferin) is not a substrate for luciferase until it is cleaved; hence there is insignificant inherent background. Furthermore, the homogeneous, coupled-enzyme format of the assay insures that any contaminating free aminoluciferin is consumed before beginning the assay (Figure 7). Any contaminating free fluorophore remains in a fluorescent assay contributing to background. The only background in this bioluminescent assay is due to the spontaneous hydrolysis of the Gly-Pro-aminoluciferin substrate. Thus the inherent background is low, resulting in large signal-to-noise ratios (Figure 4). The low background also allows for a very broad range of linearity for the assay (>3 logs of DPPIV concentration; Figures 2 and 4). The assay sensitivity allows the researcher to use less enzyme if screening for DPPIV inhibitors. We recommend using ≤ 1 ng/ml of DPPIV per well; at higher concentrations the kinetics of the assay are compromised due to product inhibition of the luciferase reaction.

The assay is not dependent on accumulation of cleaved product because the light output is a result of the luciferase consuming aminoluciferin as soon as it is produced. Maximum signal and sensitivity are achieved as soon as the DPPIV and luciferase activities reach a steady-state. Typically this occurs in 30 minutes; therefore, the assay is extremely sensitive in a short time frame.

Temperature

The intensity and rate of decay of the luminescent signal from the DPPIV-Glo™ Protease Assay depends on the rate of the luciferase reaction. Environmental factors that affect the rate of the luciferase reaction will result in a change in the intensity of light output and the stability of the luminescent signal.

Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates to a constant temperature prior to performing the assay. For batch-mode processing of multiple assay plates, precautions should be taken to ensure complete temperature equilibration. Plates removed from a 37°C incubator and placed in tall stacks at room temperature will require longer for equilibration than plates arranged in a single layer.

Chemicals

The chemical environment of the luciferase reaction will affect the enzymatic rate and thus luminescence intensity. Solvents used for various chemical compounds may interfere with the luciferase reaction and thus the light output from the assay. Dimethylsulfoxide (DMSO), commonly used as a vehicle to solubilize organic chemicals, has been tested at final concentrations up to 10% in the assay and found to have a minimal effect on light output.

Mixing

Mixing is not absolutely required after adding the DPPIV-Glo™ Reagent, although it may aid in reproducibility between wells.

6. References

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7. Related Products

Product	Size	Cat.#
Calpain-Glo™ Protease Assay	10ml**	G8501
Proteasome-Glo™ Cell-Based Assay	10ml**	G8660
Caspase-Glo® 3/7 Assay*	2.5ml**	G8090
Caspase-Glo® 8 Assay*	2.5ml**	G8200
Caspase-Glo® 9 Assay*	2.5ml**	G8210
Apo-ONE® Homogeneous Caspase-3/7 Assay	1ml	G7792
(fluorescent)	10ml**	G7790
CellTiter-Glo® Luminescent Cell Viability Assay	10ml**	G7570
Kinase-Glo® Luminescent Kinase Assay	10ml**	V6711
Kinase-Glo® Plus Luminescent Kinase Assay	10ml**	V3771
MAO-Glo™ Assay	200 assays**	V1401
Pgp-Glo™ Assay System	10ml	V3591
Pgp-Glo™ Assay System with P-glycoprotein	10ml	V3601
P450-Glo™ CYP1A1 Assay	10ml	V8751
	50ml	V8752
P450-Glo™ CYP1B1 Assay	10ml	V8761
	50ml	V8762
P450-Glo™ CYP1A2 Assay	10ml	V8771
	50ml	V8772
P450-Glo™ CYP2C8 Assay	10ml	V8781
	50ml	V8782
P450-Glo™ CYP2C9 Assay	10ml	V8791
	50ml	V8792
P450-Glo™ CYP2C19 Assay	10ml	V8881
	50ml	V8882
P450-Glo™ CYP2D6 Assay	10ml	V8891
	50ml	V8892
P450-Glo™ CYP3A4 Assay	10ml	V8801
	50ml	V8802
P450-Glo™ CYP3A7 Assay	10ml	V8811
	50ml	V8812
P450-Glo™ CYP1A2 Screening System	1,000 assays	V9770
P450-Glo™ CYP2C9 Screening System	1,000 assays	V9790
P450-Glo™ CYP3A4 Screening System	1,000 assays	V9800
P450-Glo™ CYP2C19 Screening System	1,000 assays	V9880
P450-Glo™ CYP2D6 Screening System	1,000 assays	V9890

*For Laboratory Use. **Available in larger sizes.

^(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.

^(b)U.S. Pat. No. 6,602,677 and Australian Pat. No. 754312 have been issued to Promega Corporation for thermostable luciferases and methods of production. Other patents are pending.

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