CUSTOM ENZYME SUBSTRATES FOR LUCIFERASE-BASED ASSAYS

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Promega provides a variety of custom substrates for use in luciferase-based protease assays for low-volume, high-throughput formats. Luciferase-based screening has the advantage of low compound interference and simple assay protocols that are amenable to high-throughput applications and batch plate processing.

In basic research institutions and high-throughput screening centers, bioluminescent assays are supplanting fluorescent assays due to increased sensitivity, improved robustness, and greater dynamic linearity (1). Bioluminescence is especially superior over fluorescence in low-volume formats.

A basic (native) firefly luciferase reaction requires luciferase, luciferin, ATP and oxygen for light production. Luciferase catalyzes the conjugation of luciferin to ATP and the subsequent oxidation of the luciferyl-AMP intermediate. Ultimately, the luciferase provides an environment in which the oxidized luciferin intermediate rearranges to produce oxyluciferin and a single photon with high-quantum efficiency. Although different luciferases emit light at different wavelengths, light from native firefly luciferase emits at 560nm and is easily detected by a luminometer.

Light intensity of firefly bioluminescence is correlated with the chemical concentrations of the reaction components. By holding the concentrations of all components in the luminescent reaction constant, except for one that is allowed to vary in correlation with the process of interest, light intensity can be used to measure the molecular process of interest. Depending on the assay design, light output can be proportional to any one of the required reaction components. For example in reporter gene assays, light is proportional to the concentration of luciferase. In cell viability, kinase and cAMP assays, light is proportional to the ATP concentration. In assays for proteases, cytochrome P450, glutathione-S-transferase (GST) and monoamine oxidase (MAO), a "pro-luciferin" is used to couple luciferin concentrations to the activity of a specific enzyme.

Assays of Luciferin Concentration

The pro-luciferin approach has proven useful for sensitive assays for multiple enzymes including cytochrome P450s, caspases, other proteases, MAO and GST (2–7; Figure 1). A pro-luciferin is a molecule that cannot support luminescence directly when combined with luciferase, but can be converted into luciferin through catalytic processing by a second enzyme. By this means, the luminescent signal of an assay becomes dependent on this second enzyme. Luminescent assays based on enzymatic conversions of pro-luciferins are typically 10- to 100-fold more sensitive than comparable fluorogenic assays. For protease assays, a pro-luciferin contains the target protease recognition sequence covalently attached to aminoluciferin, an alternative luciferase substrate. The substrate is combined with all of the components necessary to support luminescence. Protease cleavage of the peptidyl recognition sequence generates aminoluciferin, which is immediately used as a substrate for luciferase in a coupled reaction. Consequently, a constant luminescent signal is produced in this steady-state reaction, where the light intensity is proportional to the level of protease activity.

As an example, Z-DEVD-aminoluciferin is a pro-luciferin specific for protease cleavage by caspase-3 and caspase-7. Caspase-3/7 cleaves DEVD just after the second aspartic acid residue (D), and the resulting aminoluciferin is a luminescent substrate for the luciferase reaction. The light output from the luciferase reaction is directly proportional to the activity of the caspase enzyme. Figure 2 illustrates the superior reaction

Ultra-Glo™ rLuciferase

We engineered a superior recombinant luciferase to meet the requirements of assay conditions that the native enzyme could not withstand. The luciferin (and ATP) assays discussed here require a robust enzyme to withstand variations in temperature, concentration of ionic detergents and reducing agents. That enzyme is Ultra-Glo™ Recombinant Luciferase, a key component in all of our "Glo" luciferase-based assays.

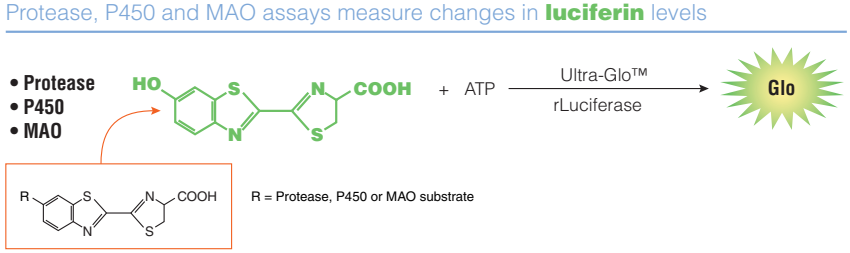

Figure 1. Bioluminescent assays based on the pro-luciferin (amino-modified) coupled reaction approach.

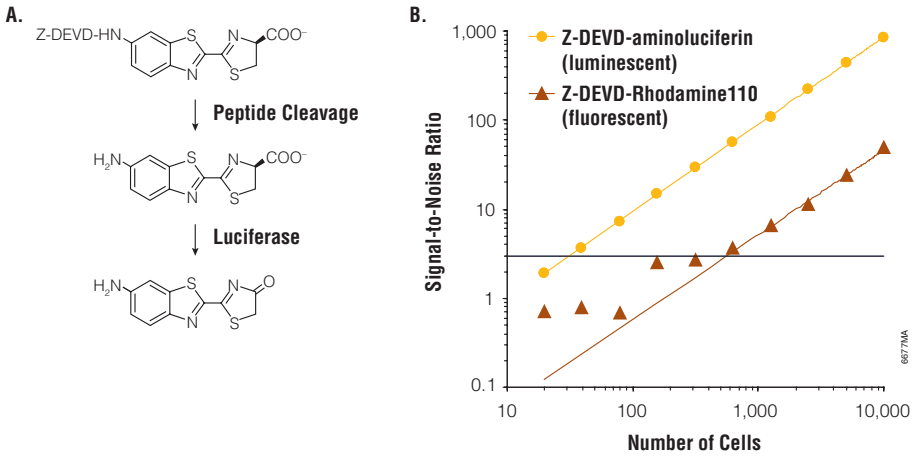

Figure 2. A comparison of bioluminescent and fluorescent measurements of caspase-3/7 activity. Z-DEVD-aminoluciferin (circles); Z-DEVD-rhodamine 110 (triangles). The assays were run simultaneously using the same anti-FAS-treated Jurkat cells serially diluted in 96-well plates. Readings were taken 1 hour after adding the substrates. Results were plotted as signal:noise (mean signal–mean background/standard deviation of background). Background signals were determined from wells containing culture medium without cells. The limit of detection is defined as the number of cells giving a signal:noise ratio ≥3 (black horizontal line). Reprinted with permission from O'Brien *et al.* (3).

dynamics of luminescence versus fluorescence when the only difference is the tag used, Z-DEVD attached to aminoluciferin or Z-DEVD attached to rhodamine 110. Caspase-Glo® 3/7 Assay, which detects general apoptosis activity, is now routinely used in industry as well as other segments of research.

We have developed a number of assays for additional caspases, such as caspase-8 and caspase-9, and for other proteases, such as calpain, dipeptidyl peptidase IV, and the proteasome. Recently our caspase-8 and caspase-9 assays were used directly on Zebrafish embryos in 96-well microplates to monitor these initiator caspases following the effects of ionizing radiation (8; see review on page 4).

Table 1 provides a representative listing of the pro-luciferin-protease substrates synthesized by Promega.

Summary

The chemical and physical advantages of luciferase-based assays over comparable fluorescent-based assays have been demonstrated in numerous articles and applications. Firefly luciferase-based assays provide an additional advantage in flexibility whereby the luciferase reaction can be coupled to another target-specific enzymatic reaction with a modified pro-luciferin substrate. The assay readout retains the same features of the native firefly luciferase reactions: sensitivity, robustness, and dynamic linearity. Finally, we have mastered the chemistry necessary to synthesize specific pro-luciferins of many types thereby widening the range of possible assays.

References

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Table 1. Enzyme Substrates and Assays Offered by Promega.

Substrate	Enzyme	Assay Available
Z-DEVD-aminoluciferin	caspases-3 and -7	Caspase-Glo® 3/7 Assay (G8090, G8091, G8092, G8093)
Z-LETD-aminoluciferin	caspase-8	Caspase-Glo® 8 Assay (G8200, G8201, G8202)
Z-LEHD-aminoluciferin	caspase-9	Caspase-Glo® 9 Assay (G8210, G8211, G8212)
GP-aminoluciferin	dipeptidyl peptidase 4 (DPPIV)	DPPIV-Glo™ Protease Assay (G8350, G8351)
Suc-LLVY-aminoluciferin	calpain- and chymotrypsin-like activities of proteasome	Calpain-Glo™ Protease Assay (G8501, G8502); Proteasome-Glo™ Chymotrypsin-Like Assay (G8621, G8622)
Z-LRR-aminoluciferin	trypsin-like activity of proteasome	Proteasome-Glo™ Trypsin-Like Assay (G8631, G8632)
Z-nLPnLD-aminoluciferin	caspase-like activity of proteasome	Proteasome-Glo™ Caspase-Like Assay (G8641, G8642)

Substrates Available by Custom Order.

Substrate	Enzyme	Substrate	Enzyme
Z-QEVY-aminoluciferin	calpain and proteasome chymotrypsin-like activity	Z-FR-aminoluciferin	cathepsins B/L
VP-aminoluciferin	dipeptidyl peptidase 4 (DPPIV)	Boc-VPR-aminoluciferin	kallikrein or thrombin
Z-VDVAD-aminoluciferin	caspase-2	Z-GGR-aminoluciferin	thrombin
Z-VEID-aminoluciferin	caspase-6	Ac-K-aminoluciferin	trypsin
Z-ATAD-aminoluciferin	caspase-12	AAF-aminoluciferin	aminopeptidase
Z-IEPD-aminoluciferin	granzyme B	Suc-AAPF-aminoluciferin	serine aminopeptidase
Z-IETD-aminoluciferin	granzyme B and caspase-6	Z-PRNK-aminoluciferin	tryptase
Z-TSAVLQ-aminoluciferin	SARS protease		
Z-VNSTLQ-aminoluciferin	SARS protease		

Key: Z = carboxylbenzyl; Suc = succinyl; Ac = acetyl; Boc = t-butyloxycarbamate; I = isoleucine; nL= norLeucine
All other capital letters are standard single-letter amino acid abbreviations.

Custom Substrate Inquiries: www.promega.com/myway/