Luminogenic Enzyme Substrates: The Basis for a New Paradigm in Assay Design

ABSTRACT Luciferin derivatives as luminogenic substrates provide the basis for a new paradigm in enzyme assay design that brings the advantages of bioluminescence—superior sensitivity, resistance to interference, and ease of use—to the enzymology researcher. Here we highlight our wide selection of bioluminescent enzyme substrates for proteases, metabolic enzymes and β-galactosidase.

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Luciferin derivatives as luminogenic substrates provide the basis for a new paradigm in enzyme assay design, and certain advantages, such as sensitivity and low background, are intrinsic to the approach.

INTRODUCTION

The advantages of bioluminescence are most commonly harnessed for assay design in two ways. First, gene reporter assays monitor changes in the concentration of the luciferase enzyme for studies of gene regulation. Second, ATP assays correlate changes in ATP concentration with changes in the light output of an ATP-dependent luciferase reaction for measuring biomass and ATPase activities (1). In a recent trend, a third approach brings the exquisite sensitivity and selectivity of bioluminescence into the sphere of traditional enzymology. Here we describe this bioluminescent technology, which offers a new assay choice for a wide variety of enzymes that include proteases, metabolic enzymes and β-galactosidase.

Enzymology relies heavily on the detection methods used to measure enzyme-catalyzed reaction products. Mass spectrometry, absorbance, radioactivity, fluorescence, chemiluminescence and bioluminescence are some of the most commonly used methods. Each method has advantages but differs substantially in terms of sensitivity, ease of use, cost and susceptibility to interference. Nevertheless, within its validated space, each method effectively correlates signal (e.g., peak area, counts, optical signal) with the enzyme activity being monitored. In most applications bioluminescence ranks very high in terms of sensitivity and ease of use (Figure 1).

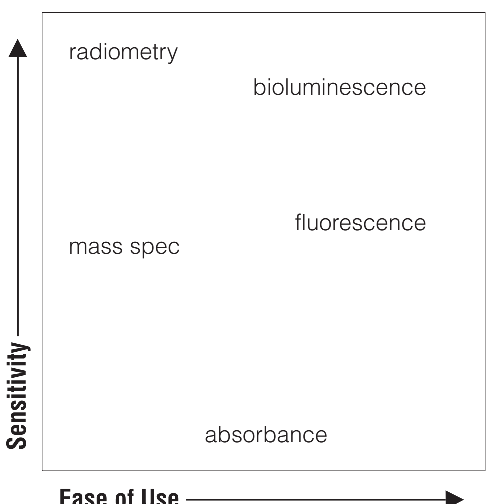

Figure 1. In most applications bioluminescence ranks very high in terms of sensitivity and ease of use.

In bioluminescence, a luciferase catalyzes the oxidation of a luminescent substrate known as a luciferin. Firefly luciferase reactions are ATP-dependent (2). Assays that use luciferase are configured by coupling a variable parameter of interest to a limiting component of the luciferase reaction (1). In the approach described here, luciferin is the limiting component. The assays are based on proluciferins, which do not react with luciferase until they are converted to a luciferin by the enzyme of interest and then

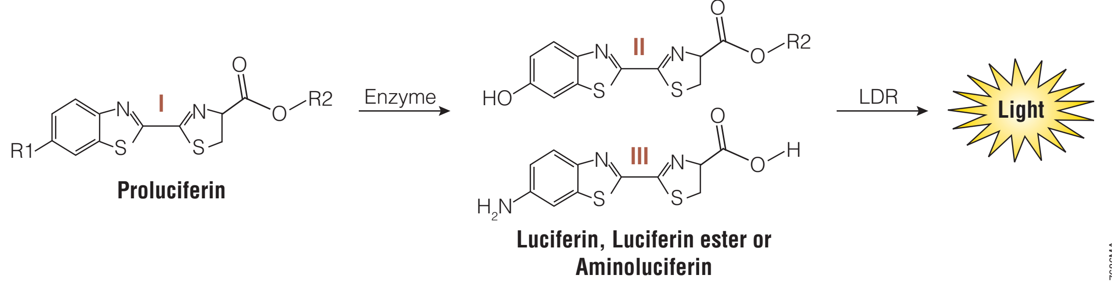

Figure 2. Luciferin derivatives as luminogenic substrates provide a new paradigm in enzyme assay design. In these bioluminescent enzyme assays, luciferin is limiting. The assays are based on luciferin derivatives (I) that are substrates for enzymes that convert them to luciferins (II and III). Luciferin is then detected in a second reaction with luciferase containing luciferin detection reagent (LDR). The amount of light correlates with the amount of luciferin produced by the activity of the enzyme in the first reaction.

detected in a second reaction with luciferase (Figure 2). The intensity of light correlates with the amount of luciferin produced and therefore with the activity of the enzyme in the first reaction.

Luciferin derivatives as luminogenic substrates provide the basis for a new paradigm in enzyme assay design and certain advantages are intrinsic to the approach. Background signals are absent from most biological systems since luciferase and luciferin are found only in bioluminescent organisms. Furthermore, bioluminescent background is minimal compared to fluorescent assay chemistries. With bioluminescence, the photon emitter is brought to its excited state by the luciferase enzyme instead of a lamp. With fluorescence-based assays, background caused by the fluorescence excitation lamp requires mitigation and includes: light scatter, fluorescent emission from unreacted probe, and fluorescence from cofactors or test compounds. By eliminating the lamp, bioluminescent assays eliminate several sources of background and interference unique to fluorescence-based assays.

We have synthesized an extensive collection of luminogenic substrates with selectivity for a wide range of enzymes (Tables 1 and 2). They are used in assays for proteases (3), β-galactosidase (4), metabolic enzymes (5) including cytochrome P450 (CYP), monoamine oxidase (MAO), N-acetyl transferase 2 (NAT-2) and glutathione by way of glutathione-S-transferase activity (GST). The substrates are used in combination with a luciferin detection reagent (LDR) that contains a unique purified recombinant form of firefly luciferase that was stabilized by directed evolution (6). The LDR formulations provide glow-style luminescent signals with typical half lives of about five hours. Both cell-based and noncell-based assays are performed in multiwell, add-only formats that are easily configured on automated platforms.

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PROTEASE ASSAYS

Protease enzymes are prime candidates for adapting luminogenic substrates because peptides can be attached via a peptide bond to aminoluciferin to provide inactive luciferase prosubstrates. Protease selectivity is dictated by

For proteases, a one-step coupled-enzyme assay where hydrolysis occurs simultaneously with the luciferase consumption of aminoluciferin improves sensitivity and efficiency.

Table 1. Bioluminescent Enzyme Assays and Screening Systems.

Substrate	Associated Assay and/or Screening System	Assay or System Features
Z–DEVD–NH … COOH	Caspase-Glo® 3/7 Assay	Cell-based or in vitro assay for caspases 3 and 7 (8), widely used for monitoring apoptosis.
Z–LETD–NH … COOH	Caspase-Glo® 8 Assay	Cell-based or in vitro assay for caspase 8, includes proteasome inhibitor to improve specificity.
Z–LEHD–NH … COOH	Capase-Glo® 9 Assay	Cell-based or in vitro assay for caspase 9, includes proteasome inhibitor to improve specificity.
Z–VDVAD–NH … COOH	Caspase-Glo® 2 Assay	In vitro assay for caspase-2, can be adapted to cell-based assay by including proteasome inhibitor and caspase-3/7 inhibitor.
Z–VEID–NH … COOH	Caspase-Glo® 6 Assay	In vitro assay for caspase-6, ideal substrate for high-throughput inhibitor screening.
GP–NH … COOH	DPPIV-Glo™ Protease Assay	In vitro assay for DPPIV, ideal substrate for high-throughput inhibitor screening.
Suc–LLVY–NH … COOH	Calpain-Glo™ Protease Assay	In vitro assay for calpain, speed is advantageous for monitoring Ca^{2+}-activated calpain, which is rapidly autoinactivated.
	Proteasome-Glo™ Chymotrypsin-Like Assay; Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay	Cell-based or in vitro assay for the chymotrypsin-like proteasome activity, eliminates the need for lysate preparation.
Z–LRR–NH … COOH	Proteasome-Glo™ Trypsin-Like Assay; Proteasome-Glo™ Trypsin-Like Cell-Based Assay	Cell-based or in vitro assay for the trypsin-like proteasome activity, eliminates need for lysate preparation, inhibitor included to improve specificity.
Z–nLPnLD–NH … COOH	Proteasome-Glo™ Caspase-Like Assay; Proteasome-Glo™ Caspase-Like Cell-Based Assay	Cell-based or in vitro assay for the caspase-like proteasome activity, eliminates the need for lysate preparation.
AAF–NH … COOH	CytoTox-Glo™, MultiTox-Glo Assays	Cell-based assay for marker protease of cell death, can be performed in multiplex with fluorogenic live-cell protease marker assay (12).

Luciferin Detection Reagent (LDR) is provided with assay kits.

peptide sequence (Table 1). Cleavage of the peptide by a protease first yields free aminoluciferin, which then is the substrate for luciferase (7).

For proteases, a one-step coupled-enzyme assay where hydrolysis occurs simultaneously with the luciferase consumption of aminoluciferin improves sensitivity and efficiency (8). Adding the protease produces a luminescent signal that increases until a steady-state between the protease and luciferase is achieved, at which point the signal typically remains constant for several hours. Light output at steady-state reflects the amount of protease activity present, and test compounds that increase or decrease the signal are scored as activators or inhibitors, respectively. This coupled-enzyme format has been applied to several proteases (Table 1), and in all cases, the assays are more sensitive than comparable fluorescent assays and more convenient for automated high-throughput applications (9).

METABOLIC ENZYME ASSAYS

For metabolic enzymes including CYPs, MAO, NAT2 and GST, a two-step endpoint design is typically used for bioluminescent assays. These enzymes feature prominently in the transformation, detoxification and elimination of numerous endogenous and xenobiotic chemicals and in adverse drug-drug interactions. In drug discovery it is important to determine if and to what extent candidate compounds inhibit or induce these activities. In the two-step assays enzyme-dependent luciferin accumulation occurs in a first step, followed by addition of an LDR that stops the enzyme activity and detects luciferin as a luminogenic signal (5,10,11).

The CYP and MAO substrates are a series of D-luciferin derivatives that provide selectivity for one or more enzymes by varying the nature of a cleavable group attached by an ether linkage to D-luciferin (Figure 2 and Table 1). The reaction product is D-luciferin or a D-luciferin ester that is

For metabolic enzymes, a two-step endpoint design is typically used for bioluminescent assays.

Table 1. Bioluminescent Enzyme Assays and Screening Systems (continued).

Substrate	Associated Assay and/or Screening System	Assay or System Features
	GSH-Glo™ Assay	Luminescent assay to detect and quantify reduced glutathione (GSH) in cells or in various biological samples (13).
	MAO-Glo™ Assay	Luminescent assay to measure monoamine oxidase (MAO) activity from recombinant and native sources (11). Useful for determining drug candidate inhibition of MAO.
	P450-Glo™ CYP3A4 Assay	Cell-based or in vitro assay for 3A isozymes of the cytochrome P450 family. Primarily used in cell-based assays to measure induction of CYP3A by drug candidate test compounds.
	P450-Glo™ CYP3A4 DMSO-Tolerant Assay; P450-Glo™ CYP3A4 Screening System, DMSO-Tolerant (Luciferin-PPXE)	Cell-based or in vitro assay for CYP3A. Assay is tolerant to DMSO. Primarily used for screening drug candidate inhibition of CYP3A.
	P450-Glo™ CYP3A4, P450-Glo™ CYP3A7 Assays (Luciferin-BE)	First-generation in vitro assay for CYP3A4 and 3A7 isozymes of the P450 family.
	P450-Glo™ CYP2C9 Assay; P450-Glo™ CYP2C9 Screening System (Luciferin-H)	Cell-based or in vitro assay for CYP2C9. Extremely selective for CYP2C9. Assay measures either induction or inhibition of CYP2C9 by drug candidate test compounds.
	P450-Glo™ CYP1A1, P450-Glo™ CYP1B1 Assays (Luciferin-CEE)	Cell-based or in vitro assay for CYP1A1. Measures induction or inhibition of CYP1A1 by test compounds. In vitro assay for CYP1B1.
	P450-Glo™ CYP1A2, P450-Glo™ CYP2C8 Assays; P450-Glo™ CYP1A2 Screening System (Luciferin-ME)	In vitro assay for CYP1A2 or CYP2C8. Also, used in cell-based assays to measure induction of CYP4A by drug candidate test compounds.
	P450-Glo™ CYP2D6 Assay; P450-Glo™ CYP2D6 Screening System (Luciferin-ME-EGE)	In vitro assay for CYP2D6. Assay can be used to measure inhibition of CYP2D6 by drug candidate test compounds.
	P450-Glo™ CYP2C19 Assay; P450-Glo™ CYP2C19 Screening System (Luciferin-H-EGE)	In vitro assay for CYP2C19. Assay used to measure inhibition of CYP2C19 by drug candidate test compounds.
	Beta-Glo® Assay System (6-0-β-galactopyranosyl-luciferin)	Homogeneous method for quantitating β-galactosidase expression in cells.

Luciferin Detection Reagent (LDR) is provided with assay kits.

Table 2. Luciferin Derivatives Available as Stand-alone Substrates.

Structure	Substrate Name	Description
	Luciferin-NAT2 Luminogenic Substrate	N-acetyltransferase 2 (NAT2) converts this weak luciferase substrate to a strong luciferase substrate (14). Useful for determining drug candidate inhibition of NAT2.
	Luciferin-3A7 Luminogenic Substrate	The 3A7 isozyme of the CYP450 family selectively converts this compound to luciferin. Useful for determining drug candidate inhibition of CYP3A7.
	Luciferin-4A11 Luminogenic Substrate	The 4A11 isozyme of the CYP450 family selectively converts this compound to a known luciferase substrate. Useful for determining drug candidate inhibition of CYP4A11 and CYP4A induction in a cell-based assay.
	Luciferin-4F2/3 Luminogenic Substrate	The 4F2 and 4F3 isozymes of the CYP450 family selectively convert this compound to luciferin. Useful for measuring CYP4F2 and CYP4F3 activity and inhibition.
	Luciferin-4F12 Luminogenic Substrate	The 4F12 isozyme of the CYP450 family selectively converts this compound to luciferin. Useful for measuring CYP4F12 activity and inhibition.
	Luciferin-2J2/4F12 Luminogenic Substrate	The 2J2 and 4F12 isozymes of the CYP450 family convert this compound to a luciferin. Useful for measuring CYP2J2 and CYP4F12 activity and inhibition.
	Luciferin-MultiCYP Generic Luminogenic Substrate	Several cytochrome P450 isozymes convert this compound to luciferin. Potentially useful for evaluating liver samples for metabolic potential or P450 structure or activity studies.

Note: These substrates are supplied as standalone lyophilized powders. They must be used in conjunction with Luciferase Detection Reagent in order to perform a luminescent assay. Please see the Product Information sheet supplied with each substrate for solubility information.

processed to D-luciferin by an esterase included in the LDR. The CYP and MAO assays are exquisitely sensitive, and they detect enzyme inhibitors as compounds that decrease light output with IC_{50}s that correlate well with conventionally determined values (5). The CYP assays also are used in a cell-based approach for measuring CYP induction by chemicals that increase light output.

Treatments that cause a decrease in cellular reduced glutathione levels (GSH) typically have a toxic effect. This can indicate the presence of a reactive electrophile, an inhibitor of GSH synthesis, or conditions of oxidative stress. A luminescent assay for measuring GSH concentration in cell lysates is configured around a luciferin derivative that is a GST substrate linked to luciferin by a sulfonate bond. GST transfers its substrate to GSH with the displacement of luciferin. Lysates are prepared from cultured cells by adding a reagent that includes GST and the luciferin derivative. The amount of GST-dependent luciferin produced is dependent on the GSH concentration, so the amount of light produced when LDR is added is proportional to the GSH concentration. Treatments that reduce light output correlate with decreased GSH levels.

The biotransformation of many xenobiotic chemicals includes acetylation by N-acetyltransferase (NAT) enzymes. The luminogenic substrate for NAT2 is an aminoluciferin derivative that reacts weakly with luciferase to give a dim luminescent signal (14). Acetylation of this substrate by NAT2 converts this derivative to a strong substrate that produces bright signals with luciferase in a two-step assay format. Of the two N-acetyl transferases expressed in humans, NAT1 and NAT2, the luminogenic substrate is highly selective for NAT2. Compounds that inhibit the light output of this assay may be non-competitive inhibitors of NAT2 or NAT2 substrates acting as competitive inhibitors.

Luciferins, the light-generating substrates of firefly luciferase, are versatile scaffolds for the synthesis of luminogenic enzyme assay substrates.

β-GALACTOSIDASE ASSAY

β-galactosidase is widely used as a reporter for gene expression studies and in complementation assays that monitor the functional assembly of two β-galactosidase fragments. 6-O-β-galactopyranosyl-luciferin (Table 1) is hydrolyzed by the β-galactosidase enzyme to produce D-luciferin that is detected in a one-step approach similar to a one-step protease assay. Although many assay systems for this enzyme exist in colorimetric and fluorescent formats, the luminogenic approach is the most sensitive and convenient (4).

SUMMARY

Luciferins, the light-generating substrates of firefly luciferase are versatile scaffolds for the synthesis of luminogenic enzyme substrates, as the present inventory of bioluminescent enzyme assays indicates. The assays bring benefits to enzymology that were previously limited mainly to assays that use luciferase as a genetic reporter or ATP sensor (2). The benefits include:

- Exquisite sensitivity.
- Homogeneous formats.
- Scalability to 96-, 384-, 1536- and 3456-well formats.
- Simple luminescent readout.
- No fluorescent interference.

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ORDERING INFORMATION

Product	Size	Cat.#
Caspase-Glo® 3/7 Assay	100 ml	G8092
Caspase-Glo® 8 Assay	100 ml	G8292
Caspase-Glo® 9 Assay	100 ml	G8212
Caspase-Glo® 6 Assay	50 ml	G0971
Caspase-Glo® 2 Assay	50 ml	G0941
DPPIV-Glo™ Protease Assay	50 ml	G8351
Calpain-Glo™ Protease Assay	50 ml	G8502
Proteasome-Glo™ 3-Substrate Cell-Based Assay System	10 ml	G1180
Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay	10 ml	G8660

Additional sizes available.

Product	Size	Cat.#
Proteasome-Glo™ Trypsin-Like Cell-Based Assay	10 ml	G8760
Proteasome-Glo™ Caspase-Like Cell-Based Assay	10 ml	G8860
MultiTox-Glo Multiplex Cytotoxicity Assay	10 ml	G9270
CytoTox-Glo™ Cytotoxicity Assay	10 ml	G9290

For Laboratory Use. Additional Sizes Available

Product	Size	Cat.#
P450-Glo™ CYP1A1 Assay	10 ml	V8751
P450-Glo™ CYP1B1 Assay	10 ml	V8761
P450-Glo™ CYP1A2 Assay	10 ml	V8771
P450-Glo™ CYP2C8 Assay	10 ml	V8781
P450-Glo™ CYP2C9 Assay	10 ml	V8791
P450-Glo™ CYP3A4 Assay	10 ml	V8801
P450-Glo™ CYP3A7 Assay	10 ml	V8811
P450-Glo™ CYP2C19 Assay	10 ml	V8881
P450-Glo™ CYP2D6 Assay	10 ml	V8891
P450-Glo™ CYP3A4 Assay (Luciferin-PPXE) DMSO-Tolerant Assay	10 ml	V8911
P450-Glo™ CYP3A4 Assay (Luciferin-PFBE) Cell-Based/Biochemical Assay	10 ml	V8901

Additional sizes available.

Product	Size	Cat.#
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
P450-Glo™ CYP3A4 Screening System (Luciferin-PPXE) DMSO-Tolerant	1,000 assays	V9910

Additional sizes available.

Product	Cat.#
Luciferin-NAT2 Luminogenic Substrate	P1721
Luciferin-3A7 Luminogenic Substrate	P1741
Luciferin-4A11 Luminogenic Substrate	P1621
Luciferin-4F2/3 Luminogenic Substrate	P1651
Luciferin-4F12 Luminogenic Substrate	P1661
Luciferin-2J2/4F12 Luminogenic Substrate	P1671
Luciferin-MultiCYP Generic Luminogenic Substrate	P1731

Product	Size	Cat.#
Luciferin Detection Reagent	10 ml	V8920
Luciferase Detection Reagent with Esterase	10 ml	V8930

Additional sizes available.

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