Chemiluminescence: sensitive detection technology for reporter gene assays

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A series of enzyme-activated chemiluminescence-based assays of reporter gene expression, useful in many biomedical applications, has been developed. The chemiluminescence detection systems for β-galactosidase, β-glucuronidase (GUS), and secreted placental alkaline phosphatase (SEAP) reporter enzymes are all based on use of 1,2-dioxetane substrates. This detection technology also permits the combined luminescence detection of two different reporter enzymes in the same tube, e.g., a dual assay for β-galactosidase and luciferase. The sensitivity of these chemiluminescence assays is several orders of magnitude greater than that of conventional colorimetric or fluorometric detection methods; e.g., the detection limit for β-galactosidase by the chemiluminescence assay is 8 fg and by a fluorometric assay is 2 pg. Furthermore, chemiluminescence enables detection of β-galactosidase, GUS, and SEAP enzyme concentrations over a dynamic range of more than five to six orders in magnitude. These assays offer highly sensitive, quantitative, rapid, nonsotopic detection of reporter enzymes that are widely used in both mammalian cells and plant cells.

INDEXING TERMS: 1,2-dioxetane • β-galactosidase • β-glucuronidase • luciferase • alkaline phosphatase • multianalyte assays

Chemiluminescent 1,2-dioxetane enzyme substrates [1, 2] are now widely used in clinical immunoassays [3, 4]. These chemiluminescence immunoassays exhibit several advantages, of which high detection sensitivity and simplicity are most important. Recently, we have developed and commercialized several assays for detecting gene expression that are based on chemiluminescent 1,2-dioxetane enzyme substrates and polymeric enhancers.

Gene expression assays, used widely in both biomedical and pharmaceutical research, are suitable for screening combinatorial chemical and natural product libraries. Reporter gene assays have also become essential in the study of gene regulation.

1 Definitions and nonstandard abbreviations: CAT, chloramphenicol acetyltransferase; β-Gal, β-galactosidase; GUS, β-glucuronidase; hGH, human growth hormone; SEAP, secreted placental alkaline phosphatase; BSA, bovine serum albumin; CSPD, disodium 3-chloro-3-(methoxyspiro[1,2-dioxetane-3,2'-{5'-chloro}tricyclo[3.3.1.1\textsuperscript{3,7}]decan]-4-yl)phenyl phosphate, chemiluminescent alkaline phosphatase substrate; Galacton, 3-(4-methoxyspiro[1,2-dioxetane-3,2'-{5'-chloro}tricyclo[3.3.1.1\textsuperscript{3,7}]decan]-4-yl)phenyl-β-D-galactopyranoside, and Galacton-Plus, 3-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2'-{5'-chloro}tricyclo[3.3.1.1\textsuperscript{3,7}]decan]-4-yl)phenyl-β-D-galactopyranoside, chemiluminescent β-galactosidase substrates; Sapphire-II, poly(benzyltributyl)ammonium chloride, and Emerald, poly(benzylidimethylboryl)ammonium chloride and sodium fluorescein, chemiluminescence enhancers.
chemiluminescence, and immunodetection methods [5]. Isotopic detection methods (for CAT and hGH reporter proteins) pose a biohazard and are limited by high cost, poor sensitivity, and narrow dynamic range [5, 18]. Colorimetric detection (β-Gal and SEAP) generally has low sensitivity [8, 13]. Fluorescence methods (β-Gal and GUS) are often limited by the presence of a high amount of autofluorescence and are subject to quenching by high amounts of protein [19]. Immunoassay detection (β-Gal, CAT, and hGH) is labor intensive and has low sensitivity. In contrast, luminescence assay methods (β-Gal, GUS, SEAP, luciferase, and aequorin) provide high sensitivity, a wide dynamic assay range, and simple assay formats [5, 8, 18, 20, 21].

We have developed a series of 1,2-dioxetane enzyme substrates (Galacton™ and Galacton-Plus™ for β-Gal, Glucuron™ for GUS, and CSPD® for alkaline phosphatase) and incorporated them into assays, Galacto-Light™ [20], GUS-Light™ [20, 21], and Phospha-Light™ [20, 21] chemiluminescence reporter gene assay systems, respectively. The Galacton-Plus substrate is also included in the Dual-Light™ [22] reporter gene assay system for detecting both β-Gal and luciferase activities in a single-tube assay. Enzymatic cleavage of 1,2-dioxetane substrates produces an unstable excited-state anion intermediate that subsequently fragments with the emission of light. These assays also include a polymeric enhancer (a water-soluble macromolecule), which is necessary for the production of an intense light signal. The polymer provides numerous hydrophobic microdomains, which sequester the anion intermediate from water molecules, thereby reducing aqueous quenching of the luminescence reaction. In the presence of these enhancer reagents, which include Emerald™ and Sapphire-II™, the signal is enhanced by ~100- to 1000-fold [23].

Luminescence reporter gene assays are ideally performed with a luminometer for maximum sensitivity, but the luminescence can also be measured with a scintillation counter [24, 25]. Here, we describe the experimental protocols and present the results obtained with these luminescence assay systems incorporating 1,2-dioxetane substrates for detecting β-Gal, SEAP, and a dual assay for β-Gal and luciferase reporter enzymes.

Materials and Methods

REAGENTS AND INSTRUMENTATION

Purified human placental alkaline phosphatase (P-3895; EC 3.1.3.1), β-Gal (G-5635; EC 3.2.1.23), and bovine serum albumin (BSA; A-3059) were obtained from Sigma (St. Louis, MO). Purified firefly (Photinus pyralis) luciferase (EC 1.13.12.7) was obtained from Analytical Luminescence Laboratories (Ann Arbor, MI). Light emission was measured in an AutoClimiLumat LB 952 T (Berthold; Wallac, Gaithersburg, MD), an ML2250 Microtiter® plate luminometer (Dynatech Laboratories, Chantilly, VA), or in a Turner Model 20e luminometer (Turner Designs, Mountain View, CA). The following are products of Tropix (Bedford, MA): Galacton and Galacton-Plus substrates for β-Gal are components of the Galacto-Light and Galacto-Light Plus kits, respectively, which also include reaction buffer and Accelerator-II. CSPD alkaline phosphate substrate, dilution buffer, assay buffer, and reaction buffer are components of the Phospha-Light kit. The Dual-Light Assay System contains lysis buffer, buffer A, buffer B, and Accelerator-II. The lysis buffers and reaction buffers are specific for each kit.

All assays are performed in triplicate, and the results plotted represent the average of triplicate determinations.

ASSAYS

β-Gal. Galacto-Light reaction buffer, 180 μL, was added to 20-μL aliquots of purified β-Gal, diluted with 10 g/L BSA solution in 0.1 mol/L sodium phosphate, pH 8. After a 60-min incubation at room temperature, 300 μL of Accelerator-II was added and the luminescence intensity was measured at 60 min as a 5-s integral.

SEAP. Purified human placental alkaline phosphatase was diluted in 0.15 mol/L NaCl containing 0.05 mol/L Tris pH 7.4. From each enzyme dilution, 100 μL was added to 100 μL of assay buffer. After the addition of 100 μL of reaction buffer, containing CSPD and Emerald enhancer, the sample was incubated for 20 min at room temperature and placed in the luminometer. Light intensities were determined as a 5-s integral.

Dual assay for β-Gal and luciferase. Purified enzymes were diluted in lysis buffer (0.1 mol/L potassium phosphate, pH 7.8, 2 mL/L Triton X-100) containing 0.5 mmol/L dithiothreitol and 1 g/L BSA. A 10-μL aliquot of diluted enzyme was added to each well of an opaque white microplate (Microlite 2™; Dynatech Laboratories). Subsequently, 25 μL of buffer A was added to each well, and the plate was placed in a luminometer. The relative light units obtained with luciferase were measured for 5 s, beginning 2 s after the injection of 100 μL of buffer B. The relative light units for β-Gal were determined 30 min after the addition of buffer B and initiated by the injection of 100 μL of Accelerator-II. Intensity of the luminescence signal was measured for 5 s after a 2 s delay.

Results and Discussion

Serial dilutions of purified β-Gal, placental alkaline phosphatase, and luciferase were assayed, and the detection limits obtained for each enzyme by the chemiluminescent reporter gene assay are shown in Table 1. These results significantly advanced the level of detection compared with other methodologies. The detection limit for β-Gal obtained with Galacton-Plus substrate (0.008 pg, Fig. 1) is much lower than the published detection limits: 100 pg with a colorimetric substrate, 2 pg with a fluorescent substrate [8]. With both chemiluminescence β-Gal substrates, the dynamic range exceeds five orders of magnitude (Fig. 1) [20].

An important benefit of the Galacton-Plus substrate is the prolonged light-emission kinetics. With Galacton-Plus, the signal intensity remains constant for ~1 h after addition of the light-emission accelerator [20]. The longer-lived signal allows the use of a variety of instruments, particularly luminometers and scintillation counters without injectors. Both β-Gal assays use reaction buffers at pH 8, which allows discrimination between the bacterial reporter enzyme and the endogenous
mammalian enzyme [8]. In addition, Glucuron substrate allows detection of as little as 60 pg of purified GUS, and the linear assay range extends from $10^{-13}$ to $10^{-7}$ g of enzyme [21].

Measurement of SEAP differs from that of β-Gal in that the former is detected directly in an aliquot of culture supernate, leaving the cells intact for further experimentation. In addition, the SEAP assay exhibits glow-type emission kinetics [20, 21], so this enzyme can be measured in luminometers without injection devices or scintillation counters. With CSPD substrate, one can detect 0.033 pg of purified human placental alkaline phosphatase, as much as 100-fold less than that reported for the colorimetric substrate, p-nitrophenyl phosphate (10 pg) [5]. The dynamic range of measurement covers five orders of magnitude of enzyme concentration (Fig. 2). Because placental alkaline phosphatase is thermally stable, a simple heat treatment inactivates any nonplacental alkaline phosphatase isoenzymes present in the samples [13, 21]. L-Homoarginine further inhibits nonplacental enzyme activity [13, 21].

The ability to detect both luciferase and β-Gal activity in a single extract aliquot is a unique feature of the Dual-Light assay system. The assay system was designed to be more convenient without sacrificing the sensitivity of the individual assays and to reduce pipetting errors. The system combines an enhanced luciferase reaction (measured immediately) with the Galacton-Plus reaction (measured 15–60 min later, immediately after the injection of Accelerator-II). The detection limit for the luciferase reaction component of the Dual-Light assay system (2 fg of luciferase) is comparable with that obtained with a single luciferase assay. The detection limit for β-Gal (0.008 pg) assayed with Galacton-Plus is similar in both the individual and the dual-enzyme detection systems [22].

Chemiluminescence quantification of β-Gal has been successfully utilized in several research applications, including analysis of mammalian cultured cell extracts [26–30], tissue extracts from transgenic animals [31–33], yeast [34] and bacterial cell extracts [35], coliform detection [36], detection of enzyme synthesized in an in vitro translation system [37], and quantitative yeast two-hybrid system assays [38, 39]. The prolonged light-emission kinetics obtained with the Galacton-Plus substrate further simplifies this assay for use in high-throughput reporter screening methods [40], and continued innovation in substrate design will yield even simpler assay formats.

The Dual-Light reporter gene assay system for quantifying both β-Gal and luciferase activity can be used either as a convenient assay for simultaneous transfection normalization and experimental determination or to multiplex experimental reporter gene assays to increase screening efficiency.

### Table 1. Detection limits for reporter gene assays.

<table>
<thead>
<tr>
<th>Reporter enzyme</th>
<th>Chemiluminescence</th>
<th>Colorimetry</th>
<th>Fluorescence</th>
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<tbody>
<tr>
<td>β-Galactosidase</td>
<td>0.03g/ONPG [8]</td>
<td>2g/MUG [8]</td>
<td></td>
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<tr>
<td></td>
<td>0.005g/Galacton</td>
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<td></td>
<td>0.008g/Gal-Plus</td>
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<td></td>
<td>0.005g/Gal-Plus,</td>
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<tr>
<td>Alkaline phosphatase</td>
<td>0.035/ONPG [13]</td>
<td>10g/pNPP</td>
<td></td>
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<tr>
<td>(placental)</td>
<td>0.0035/CSPD</td>
<td></td>
<td></td>
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<tr>
<td>Luciferase</td>
<td>0.0025/luciferin,</td>
<td></td>
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<tr>
<td></td>
<td>0.001/luciferin,</td>
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* Detection limit defined as signal/noise = 2.
* Detection limit defined as background signal + 2 SD.
* Detection limit definition not provided in reference.

MUG, methylumbelliferyl-β-o-galactopyranoside; ONPG, o-nitrophenol-β-o-galactoside; pNPP, p-nitrophenyl phosphate; NA, not available.
Chemiluminescence quantification of SEAP has been used both for research applications, including analysis of culture medium from stably transformed cells [41] and analysis for nonsecreted placental alkaline phosphate in cell extracts [27, 28, 30], and for high-throughput screening applications, including analysis of nuclear hormone receptors2 and regulation of tumor promoter, growth factor, and cAMP response elements [17] in stably transformed cells. SEAP reporter gene constructs bearing signal transduction response elements are commercially available.

In conclusion, the assays described are model assays for sensitive detection of reporter gene products. Cell-based reporter gene assays are valuable screening methods for identifying small molecules that can alter the activity of transcription factors and affect gene regulation. Transcription factors and gene expression are important targets in drug discovery efforts for important clinical conditions such as viral infection, cancer, inflammation, immune dysfunction, and hormone response. Chemiluminescent reporter gene assays provide highly sensitive detection with simple assay formats that are amenable to automation with a broad range of instrumentation for high-throughput compound screening.

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References


