

Luminometry for *In Vivo* and *In Vitro* Reporting of Firefly Luciferase

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The Turner Designs Model 20 Luminometer is a sensitive, versatile and economical luminometer designed specifically for use with firefly luciferase. Our results show that the Model 20, a direct current luminometer, is equivalent in sensitivity to a quality luminometer that employs photon-counting to quantify light emission. In addition, the Model 20 has a unique sample compartment that is adaptable to different sample tube sizes and optical filters, and allows for direct measurements of luminescence in living cells and tissues.

Introduction

The cDNA encoding luciferase (*luc*) was cloned from the North American firefly *Photinus pyralis* in 1985 (1) and is now emerging as the gene of choice for *in vitro* and *in vivo* reporting of transcriptional activity in eukaryotic cells. Luciferase generates luminescence through mono-oxygenation of beetle luciferin, utilizing O₂ and ATP as cosubstrates. The modified *in vitro* luciferase reaction, containing coenzyme A, produces constant light intensity which gradually decreases after 30 seconds (2). Because light output is linearly proportional to the amount of luciferase, luminescence correlates directly to expression of the *luc* reporter gene in transfected cells or cell lysates.

Luminometers provide the most convenient, versatile and sensitive means of quantifying light emission from the luciferase reaction. Several models of luminometers exist differing in price, design and features, which can make instrument selection especially difficult for users new to the technology. Although most standard liquid scintillation counters can accommodate the luciferase assay (3), they typically do not provide the detection sensitivity nor dynamic range of a quality luminometer, and the method of sample handling is cumbersome and time consuming.

In our research at Promega, we found the Turner Designs Model 20 Luminometer to be well suited for genetic reporter applications. The instrument is sensitive and reliable and has a sample compartment design which allows flexible use. In addition, the Model 20 Luminometer is inexpensive when compared to other luminometers of similar high quality.

Photon detection

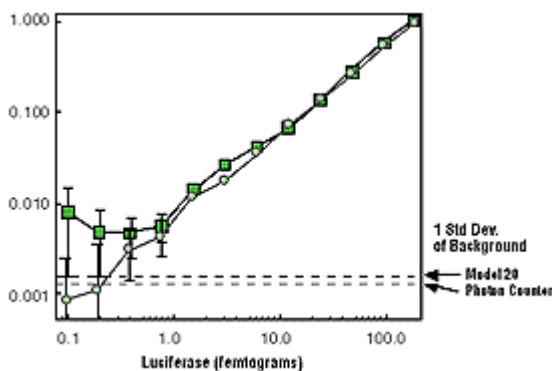
The most sensitive luminometers utilize photomultiplier tubes to detect photons, but they may differ substantially in the methods they employ to process and analyze signal input. Two different processing designs are presently found in commercial luminometers. As their names imply, "photon counting" luminometers count individual photons, while "direct current" luminometers measure electrical current that is maintained by, and is proportional to, the photon flux passing through the photomultiplier tube. Both designs allow the measurement of ultra-low light emission.

Quality luminometers detect light intensity over more than six logs and luciferase down to femtogram amounts. A common misconception is that photon-counting luminometers are more sensitive than direct current models because photon-counting models apparently detect individual photons. All including photon-counting devices, are limited by their *efficiency* of photon detection, which is influenced by numerous factors both internal (e.g., electronic and optical design) and external (e.g., sample type, volume and presentation). In the case of photon-counting luminometers, while every count is the result of a photon, not every photon is counted.

Because of the many factors that influence luminometer sensitivity, the sensitivity of an instrument's photon detection mechanism should be tested empirically. The linear range and precision of measurement should also be tested. Generally, the linear range of photon-counting luminometers is limited by the rate at which electronic pulses from the photomultiplier tube can be detected.

Comparison of luminometer sensitivities

The sensitivity of the Turner Designs Model 20 Luminometer, which utilizes a direct current design for photon detection, was compared to a popular photon counting luminometer. [Figure 1](#) shows the results of measuring a dilution series of purified luciferase using the Promega Luciferase Assay System (Cat.# E1500). To allow a direct comparison of the values reported by each instrument, the relative light units of each were normalized to a common scale. This was done by first determining the luminometer reading for the luminescence reaction with no luciferase added and subtracting this value from all other readings, then adjusting the highest reading (200 femtograms) to a value of 1. The results are shown using bilogarithmic scales to reveal the relative deviations from linearity.



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Figure 1. Sensitivity comparison between the Turner Designs Model 20 (direct current) Luminometer and a photon-counting luminometer. Firefly luciferase was sequentially diluted 2-fold in Promega's 1X Cell Culture Lysis Reagent (CCLR) supplemented with 1mg/ml BSA and held on ice. The luciferase assay was initiated by manually adding between 200 and 0.098 femtograms of luciferase in a 10 μ l volume to 100 μ l of Luciferase Assay Reagent (LAR), vortexing briefly then quantifying light emission over a 10-second period. Reactions were carried out in cuvettes recommended by the respective luminometer manufacturers: 5ml (12mm) polystyrene cuvettes for the photon-counting luminometer (squares) and 1.6ml (8mm) polypropylene tubes for the Model 20 Luminometer (circles). Light intensity values for each luciferase dilution was calculated from the average of 5 reactions, except for the 4 greatest dilutions where 10 reactions were used to compensate for the reduced precision of measurement. The values from each luminometer were normalized to a common scale by first subtracting the background luminescence from each averaged value, then adjusting the relative luminescence such that 200 femtograms of luciferase yielded 1 relative light unit. The background signal for

each luminometer was determined as the average reading from 20 individual reactions containing only LAR. The error bars show 1 standard deviation of individual measurements for the 4 most dilute enzyme concentrations. Precision of the baseline for each luminometer, reported as 1 standard deviation of the individual background measurements, is shown as dotted lines.

The plot does not show the entire linear range of either luminometer, rather only the most sensitive range. The results show that the linear ranges of both instruments extend to below 1 femtogram of luciferase (10,000 molecules). Below this range, we typically found that the signal of the photon-counting luminometer leveled off despite our adjustment of all values for background luminescence. We are not certain about the cause for this, though we believe it may be due to static electricity. The signal from the Model 20 Luminometer continued towards zero with greater enzyme dilution, the greatest dilution corresponding to less than 1,000 molecules of luciferase. The precision of both luminometers decreased substantially at the lowest enzyme concentrations. At higher enzyme concentrations the coefficient of variation was about 3-5%. This experiment was repeated several times with analogous results for both luminometers.

We also measured the statistical fluctuation of the baseline for both instruments since sensitivity depends on the ability to distinguish signal from baseline. Twenty independent luminescent reactions containing no luciferase were measured, and the average and standard deviation were calculated. Our results showed the baselines of both luminometers to be approximately the same; the small difference shown in [Figure 1](#) is not significant.

Measurement of *In Vivo* luminescence

An exciting and unique application of firefly luciferase is as an *in vivo* reporter of gene expression. It is possible to treat living cells with bio-active agents (e.g., chemical inducers or biological regulators of gene expression), biohazardous agents (e.g., environmental contaminants, potential chemotherapeutic drugs or antibiotics) or altered environmental conditions (e.g., temperature or osmotic shock, circadian effectors, oxygen-debt, or electrical fields) and assess their effects on physiological response by monitoring real-time changes in *luc* reporter gene expression.

The unique sample compartment design of the Turner Model 20 Luminometer permits luminescent analysis of samples in a variety of formats, including transformed cells and tissues. The open architecture of sample compartment accepts sample cuvettes, tubes or vials of sizes up to 28mm outer diameter and 60mm in height. The height can be extended to 100mm using the oversized cover. The photomultiplier tube is placed at the bottom of the sample chamber, rather than at the side as in many other instruments, and is protected from ambient light by an electronically activated shutter. Other machines typically use a mechanical shutter mechanism and can accept only one type of sample tube.

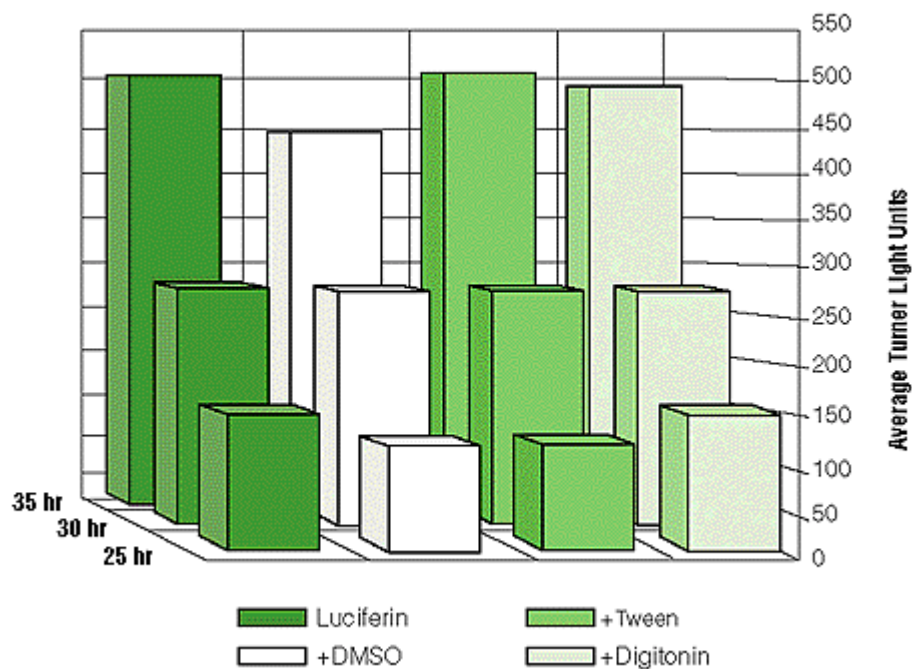
Adherent cells cultured in flat-bottom vials can be easily and rapidly analyzed for luciferase expression in the Model 20 Luminometer. These luciferase assays do not destroy the cells and are performed aseptically in culture vials, allowing for repeated analyses on the same cell cultures over time. This feature provides great flexibility in experimental design and eliminates the problem of compensating for variable transfection efficiencies between cell populations within an experimental group.

The utility of luciferase as a reporter of *in vivo* gene regulation has been controversial due to confusion about whether luciferin can permeate cell membranes. At neutral pH, luciferin carries a single negative charge, and it was presumed *a priori* that the molecule could not cross the hydrophobic lipid bilayer. Several reports, however, describe measurements of *in vivo* luminescence in animal, plant, and bacterial cells and tissues. Sometimes acidic buffers (4) or agents such as DMSO (4,5), nigericin (5), ionic

detergents (4,6) or combinations, were used to facilitate the intracellular accessibility of luciferin.

Figure 2 demonstrates both the utility of the Model 20 Luminometer in quantifying *in vivo* luminescence and the ability of exogenous luciferin to rapidly permeate cultured mammalian cells. NIH3T3 cells were transfected with Promega's pGL2-Control Vector (Cat.# E1611), a plasmid which carries the *luc* gene flanked by SV40 promoter and enhancer regions. These cells were grown in neutral medium (pH 7.2) supplemented with either luciferin alone, or in combination with DMSO, Tween® 20 or digitonin. The concentration or type of permeabilizing agent did not enhance the cellular luminescence above that observed by adding luciferase alone. All culture treatments produced similar *in vivo* luminescence which increased in proportion to the culture time.

Figure 3 shows that *in vivo* luminescence of transfected NIH3T3 cells reaches its maximum level between 30 and 45 seconds following the addition of luciferin, demonstrating that luciferin can rapidly permeate the cellular membranes.



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Figure 2. Use of the Turner Designs Model 20 Luminometer to measure the cellular accessibility of luciferin exogenously applied to transfected mammalian cells. Luciferase reporter plasmid pGL2-Control Vector was introduced into NIH3T3 cells using Promega's ProFection™ System reagents (Cat.# E1200). A modified procedure was developed in which calcium phosphate-treated DNA is added to trypsinized cells in suspension, which then were dispensed in equal volumes into individual culture plates or vials. The modified procedure is much more rapid and easier to perform, and eliminates the need to control for variable transfection efficiency within an experiment. Briefly, 1.8ml of 250mM CaCl₂ containing 30µg of pGL2-Control DNA were added in a dropwise fashion to 1.8ml of 2X HEPES buffer during vortexing. NIH3T3 cells were cultured in Dulbecco's Modified Eagles Medium supplemented with 10% calf serum (DMEM/CS) until approximately 80% confluence was observed. Cells were then trypsinized and collected in 10ml of DMEM/CS. Cells (3.0×10^6) were transferred to a 50ml Corning tube and DMEM/CS was added to obtain a final volume of 33ml. The entire 3.6ml volume of colloidal DNA/calcium phosphate was added to the cell suspension and rapidly mixed. Aliquots (3ml) were immediately dispensed into each of twelve flat-bottom borosilicate vials

(28mm O.D. with threaded caps, e.g., Fisherbrand #03-339-21J). The culture vials, each containing the equivalent of 2.5×10^5 cells and $2.5\mu\text{g}$ of pGL2-Control Vector, were incubated at 37°C , 5% CO_2 for 20 hours prior to exchanging the medium with 1ml of fresh DMEM/CS. At 25, 30 and 35 hours post-transfection of trypsinized cells, each culture was treated with DMEM/CS containing 0.5mM luciferin, or 0.5mM luciferin in combination with either 1% DMSO, 0.01% Tween-20, or 0.0005% digitonin. *In vivo* expression of the luciferase reporter gene was determined by placing individual culture vials in the Turner Designs Model 20 Luminometer sample chamber and quantifying cellular light emission over a 90-second period. The supplemented medium was then immediately exchanged for 1ml of fresh DMEM/CS and the culture vial was returned to the 37°C incubator.

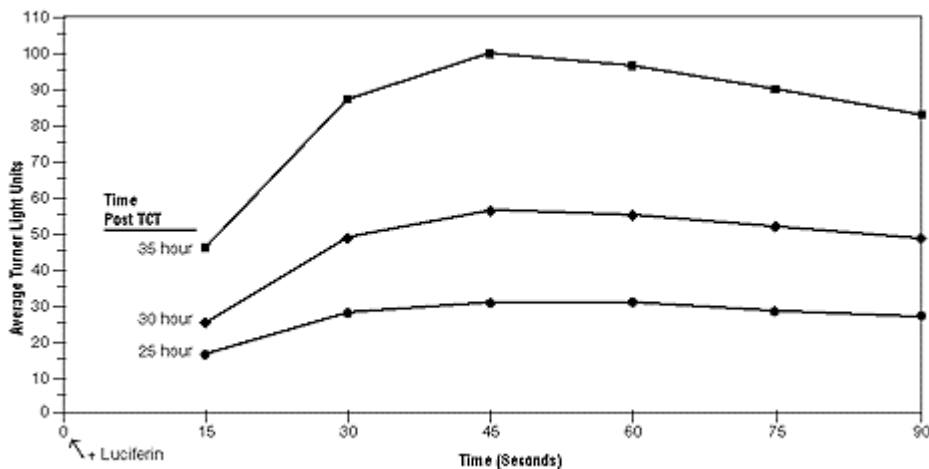


Figure 3. Time course of *in vivo* light emission from luciferin-treated NIH3T3 cells transiently expressing the luciferase reporter gene. Trypsinized NIH3T3 cells were transfected with the luciferase reporter plasmid pGL2-Control Vector, dispensed into 28mm borosilicate vials and cultured as described in Figure 2. The same culture vials were sequentially supplemented with DMEM/CS containing 0.5mM luciferin at 25, 30 and 35 hours post-trypsinized cell transfection (Post-TCT). Cellular light emission was quantified over 10-second integration periods ending at 15, 30, 45, 60, 75 and 90 seconds after the initial addition of luciferin to each culture vial.

Summary

The sensitivity, dynamic range and ease of operation of the Turner Designs Model 20 Luminometer were evaluated critically for quantifying standard luciferase assays which do not require automated injection devices. In all cases, the Model 20 Luminometer met or exceeded the performance of a popular photon-counting luminometer. In addition, the Model 20 Luminometer is the only luminometer with a versatile sample chamber compartment which allows the use of optical filters and a variety of sample tube sizes and configurations.

Measurement of luciferase activity in living cells and tissues can be performed rapidly using the Model 20 Luminometer. Assays of luciferase expression in transfected mammalian cells are accomplished easily by culturing the cells in 28mm borosilicate vials for desired intervals of time, adding luciferin, then quantifying luminescence in the Model 20 Luminometer. Tests with NIH3T3 cells transfected with pGL2-Control Vector demonstrated that luciferin uptake is rapid and does not require chemical agents to perturb the membrane structure, and likely the physiological state, of the living cells.

References

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Ordering Information

Product	Cat.#
Turner Designs Luminometer Model 20 Genetic Reporter Instrumentation Package for Stabilized Assays	E4091

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