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A single secreted luciferase-based gene reporter assay

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ABSTRACT

Promoter analysis typically employs a reporter gene fused to a test promoter combined with a second reporter fused to a control promoter that is used for normalization purposes. However, this approach is not valid when experimental conditions affect the control promoter. We have developed and validated a single secreted luciferase reporter (SSLR) assay for promoter analysis that avoids the use of a control reporter. The approach uses an early level of expression of a secreted luciferase linked to a test promoter as an internal normalization control for subsequent analysis of the same promoter. Comparison of the SSLR assay with the dual luciferase reporter (DLR) assay using HMGCR (3-hydroxy-3-methylglutaryl-coenzyme A reductase) and LDLR (low-density lipoprotein receptor) promoter constructs, which are down-regulated by 25-hydroxycholesterol, show that both assays yield similar results. Comparison of the response of the HMGCR promoter in SSLR transient assays compared very favorably with the response of the same promoter in the stable cell line. Overall, the SSLR assay proved to be a valid alternative to the DLR assay for certain applications and had significant advantages in that measurement of only one luciferase is required and monitoring can be continuous because cell lysis is not necessary.

Gene reporter assays are widely used to study the composition and activity of promoters under different experimental conditions. In such assays, the reporter gene is placed under the control of a promoter of interest and serves as a quantitative readout of promoter activity. These assays have a very high utility and are commonly used for mapping promoters and investigating the response of promoters to transcription factor activity or upstream signaling pathway activity. Several reporter genes encode enzymes whose activity can be readily and easily monitored in a quantifiable fashion. Luminescent reporter gene assays using luciferases are arguably the most prominent for promoter analysis in cell culture models and in biomolecular cell screening. The high signal-to-noise ratio and the wide dynamic range of luminescent reporter gene assays make them particularly suited for high-throughput screening applications, and the development of modified or use of novel luciferases has greatly extended the utility of these assays [1,2]. The recent development of secreted luciferases [3,4] has removed the requirement for cell lysis and has extended the utility of these assays even further, enabling continuous monitoring of promoter activity in a single sample.

The employment of luminescent reporter gene assays for investigation of promoters is typically performed by transient transfection of a promoter–luciferase construct into a host cell and measurement of luciferase activity in control and test samples under different conditions. Because transient transfection efficiencies can vary across samples, this approach requires an internal transfection control in each sample for normalization purposes. In addition to normalizing for transfection efficiencies, the internal transfection control also normalizes for other variables, including variations in cell plating and cell lysis efficiencies, toxicity, and pipetting inconsistencies [5].

A dual luciferase reporter (DLR)[1] assay system has been developed for the use of promoter–luciferase constructs in transient transfections [6] and is widely used. In this dual system, one luciferase, whose activity can be determined using a specific substrate, is used for reporting on the test promoter, whereas a second luciferase, whose activity can be determined using a different substrate and which is linked to a constitutively active promoter (e.g., that of thymidine kinase [TK], cytomegalovirus [CMV], or simian virus 40 [SV40]), functions as the control. This DLR assay system typically employs *Firefly* (*Photinus pyralis*) luciferase as the reporter for the promoter being tested and *Renilla* (*Rotylenchulus reniformis*)

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[1] *Abbreviations used:* DLR, dual luciferase reporter; TK, thymidine kinase; SSLR, single secreted luciferase reporter; FBS, fetal bovine serum; 25-OHC, 25-hydroxycholesterol; LDS, lipid-depleted serum; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; PCR, polymerase chain reaction; LDLR, low-density lipoprotein receptor; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ANOVA, analysis of variance; GLuc, luciferase from *Gaussia*; SREBP, sterol response element binding protein; ER, endoplasmic reticulum.

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luciferase as the reporter for the control promoter. *Firefly* and *Renilla* luciferases both generate light but use distinct substrates (d-luciferin and coelenterazine, respectively), which enables the activity of each enzyme in a single sample to be measured [6]. More recently, a DLR assay that uses secreted luciferases from *Cypridina* and *Gaussia* species has been developed [7]. The sensitivity and ease of measurement of this dual secreted luciferase assay is comparable to that of the original DLR assay, but it has advantages in that cell lysis is not required and readings can be taken across multiple time points from the same cell population. Like the original DLR assay, this method also requires normalization to a control reporter plasmid [7]. However, the secreted dual assay has not been widely adopted, largely due to the instability of the *Cypridina* substrate [8]. One concern with the DLR assay system is that an assumption is made that the transfection efficiency of the test plasmid is tightly correlated with the transfection efficiency of the control plasmid. In addition, the utility of the DLR assay is limited in some situations where the promoter driving the control reporter is affected by experimental conditions [9,10]. Thus, the use of the control reporter in the DLR assay system requires preliminary investigation to ensure that the control promoter is not affected by the test conditions. If the control promoter is affected by the test conditions, this obstacle may be overcome in some cases through the use of alternative control promoters; however, in many cases, factors that affect one control promoter also affect other control promoters [11].

Here we report and validate a single secreted luciferase reporter (SSLR) assay approach for promoter analysis that avoids the use of a control reporter. This assay provides a valid alternative to the DLR assay, particularly for situations where experimental factors affect control promoters. In addition, the assay offers a simpler approach than the DLR assay and can be used to replace it in appropriate situations.

Materials and methods

Culture media

HeLa cells were maintained in medium A: RPMI 1640 (Sigma) and 10% fetal bovine serum (FBS; Sigma) (37 °C, 5% CO_2). For incubation with 25-hydroxycholesterol (25-OHC; Avanti Polar Lipids), cells were switched to medium B: RPMI 1640 and 5% lipid-depleted serum (LDS). LDS was prepared by incubating 100 ml of FBS with 2 g of fumed silica (Sigma), swirling overnight at room temperature. The LDS was clarified by centrifugation at 2000*g* for 10 min at 10 °C. The supernatant was sterile filtered using 0.45-μm filter, aliquoted, and stored at −20 °C. Cholesterol content was assayed using a cholesterol assay kit (Audit Diagnostics, Ireland) and showed that the LDS was more than 99% depleted of cholesterol compared with FBS.

Cloning

The HMGCR (3-hydroxy-3-methylglutaryl-coenzyme A reductase) promoter (−270 to +77) was amplified from human DNA by polymerase chain reaction (PCR) and cloned into both the *Firefly* luciferase reporter plasmid pGL3-Basic (Promega) and the *Gaussia* luciferase plasmid pGLuc-Basic (New England Biolabs) using *Eco*RI and *Hin*dIII restriction sites. The LDLR (low-density lipoprotein receptor) promoter (−590 to +93) was also amplified from human DNA by PCR and cloned into pGL3-Basic vector using *Bgl*II and *Hin*dIII restriction sites and into pGLuc-Basic vector using *Eco*RI and *Hin*dIII restriction sites. Cloned promoter sequences were verified by DNA sequencing.

DLR assay

HeLa cells were seeded at 4×10^4 cells in 500 μl of medium A per well in a 24-well plate (37 °C, 5% CO_2). After 18 to 24 h of incubation, cells were transfected for 6 to 9 h with 450 ng of reporter plasmid and 50 ng of control reporter plasmid (pRL-TK, Promega) using 1 μl of TurboFect (Thermo Scientific) in 100 μl of serum-free medium according to the manufacturer's instructions. Following transfection, medium A was replaced with 500 μl of medium B and incubated for a further 6 h. Then 25-OHC was added to a final concentration of 1.25 μM and incubation was continued for a further 16 h. Cells were harvested by aspirating medium and washing once in ice-cold phosphate-buffered saline (PBS) and then were incubated by shaking for 30 min in 100 μl of Passive Lysis Buffer (Promega) at room temperature. Then 10 μl of each sample was removed and assayed for *Firefly* and *Renilla* luciferase activity using a microplate luminometer. The substrates used (luciferin and coelenterazine for *Firefly* and *Renilla* luciferases, respectively) were purchased from Nanolight Technology, and the DLR assay buffers were prepared from individual components essentially as described previously [12].

Firefly luciferase assay buffer contained 25 mM glycylglycine, 15 mM K_2PO_4 (pH 8.0), 4 mM ethyleneglycoltetraacetic acid (EGTA), 15 mM $MgSO_4$, 0.1 mM coenzyme A, 1 mM dithiothreitol (DTT), 2 mM adenosine triphosphate (ATP), and 75 μM luciferin. *Renilla* luciferase assay buffer contained 1.1 M NaCl, 2.2 mM ethylenediaminetetraacetic acid (EDTA), 220 mM K_2PO_4 (pH 5.1), 0.44 mg/ml bovine serum albumin (BSA), 1.3 mM NaN_3, and 1.43 μM coelenterazine. Stock solutions for all of the above were prepared in water except for coelenterazine, which was prepared in methanol and kept protected from light.

SSLR assay

Transfection was as described for the DLR assay. Total amount of plasmid DNA transfected was maintained at 500 ng and was composed of reporter plasmid or a combination of reporter plasmid and empty vector plasmid. Following transfection, medium was replaced with 500 μl of medium B. Small aliquots of medium were removed at intervals and stored at −20 °C until assayed for luciferase activity. 25-OHC treatment was as described for the DLR assay. Samples (10 μl) were assayed for *Gaussia* luciferase activity using a microplate luminometer in the *Renilla* luciferase assay buffer. A simple *Gaussia* luciferase assay buffer (PBS with 1.43 μM coelenterazine) was also tested and yielded similar results.

MTT assay

Following removal of the medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to a final concentration of 0.5 mg/ml and incubated for 2 h at 37 °C. Medium was aspirated and formazan crystals were solubilized in 150 μl of dimethyl sulfoxide (DMSO, Sigma) per well. Aliquots (100 μl) of each sample were removed to a clear 96-well plate (Sarstedt), and absorbance at 570 nM was measured.

Cell lines

HeLa cells were seeded in medium A in 10-cm dishes. Cells were transfected at 80 to 90% confluency with 15 μg of pGLuc-prom-HMGCR using 30 μl of TurboFect reagent in 3 ml of serum-free medium. Medium A was renewed after 6 h of incubation. Cells were trypsinized 48 h post-transfection and reseeded at different dilutions in medium A supplemented with 400 μg/ml G-418 (Sigma). Every 3 or 4 days, G-418/medium A was renewed until