Normalization of Luciferase Reporter Assays under Conditions that Alter Internal Controls

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The functional characterization of transcriptional regulators often requires the use of transient reporter assays. In these types of experiments, it is necessary to monitor the transfection efficiency to normalize experimental data. A number of methods have been described that utilize simultaneous transfection of reporter and internal control plasmids. Normalization for the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) relies on the pRL-TK plasmid that expresses the *Renilla* luciferase gene at low levels from a minimal herpes simplex thymidine kinase promoter (1). While the use of this reporter gene as an internal control is adequate for most experimental conditions, a number of previously published reports have demonstrated a drawback of measuring the cumulative enzymatic activity of a gene product to determine transfection efficiency (2–4). It was shown that pRL-TK is responsive to transcriptional co-activators and androgenic compounds, making it unsuitable for internal standardization when these and likely other factors are present (2–4). Here we report the use of flow cytometry to detect expression of the enhanced GFP (EGFP) under the control of a cytomegalovirus promoter to monitor transfection efficiency despite transcriptional activation by the transcription factor skNAC (5).

The muscle-specific transcription factor skNAC was previously reported to activate the myoglobin promoter upstream of a luciferase reporter (5). In the course of studying skNAC transactivation using the Dual-Luciferase Reporter Assay System, it was observed that skNAC increased *Renilla* luciferase expression from the pRL-TK internal control plasmid nearly 9-fold (data not shown). The nature of this activation is not fully understood, as skNAC significantly activated reporter genes under the control of six different promoters, including those that were completely devoid of consensus skNAC binding sites (data not shown). Therefore, a suitable method to control for transfection efficiency was sought to eliminate the search for an skNAC-unresponsive promoter. To determine transfection efficiency, a fluorescence-activated cell sorter-based method was employed. The mouse embryo fibroblast cell line C3H10T1/2 (ATCC, Manassas, VA, USA) was co-transfected with a plasmid expressing EGFP (pEGFP-N2; BD Biosciences Clontech, Palo Alto, CA, USA) and various luciferase reporter constructs using the FuGENE6™ reagent (Roche Applied Science, Indianapolis, IN, USA)

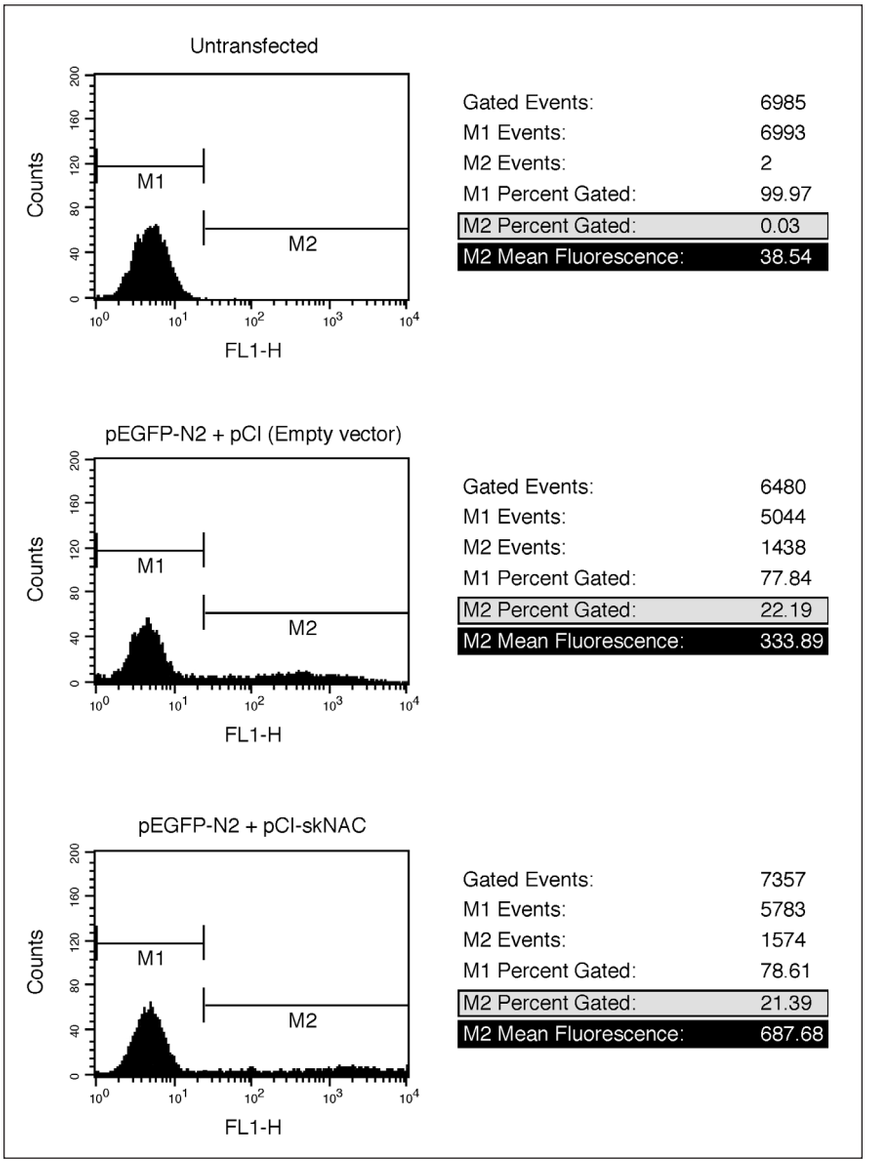

Figure 1. The use of EGFP to determine transfection efficiency. Flow cytometry analysis was performed with untransfected 10T1/2 cells (top panel) and 10T1/2 cells transiently co-transfected with pEGFP-N2 (0.5 µg) and pCI (empty vector, 3 µg; middle panel) or pEGFP-N2 (0.5 µg) and pCI-skNAC (3 µg; bottom panel). The percentage of fluorescent 10T1/2 cells was calculated using flow cytometry analysis. After gating, a marker (M1) was set according to cells not transfected with pEGFP-N2 (background fluorescence). A second marker (M2) was set for events corresponding to fluorescence above background. The number of gated events occurring within M2 divided by the total gated events yielded the percent transfection (gray bar). The levels of fluorescence (M2 mean fluorescence) of 10T1/2 cells receiving pEGFP-N2 with pCI (middle panel) or pCI-skNAC (bottom panel) are shown with black bars.

according to the instructions of the manufacturer. Twenty-four hours after transfection, cells were harvested, washed with 1 mL PBS, and resuspended in 600 µL PBS. Half of the suspended cells were centrifuged at 16 000× *g* for 30 s, resuspended in 100 µL lysis assay buffer, and frozen at -80°C for later analysis of luciferase activity using the Luciferase Assay System (Promega) according to the manufacturer's directions. The remaining cells were put on ice and analyzed by flow cytometry to determine transfection efficiency.

Flow cytometry to detect EGFP was performed using a FACSCalibur™ cytometer (BD Biosciences, San Jose, CA, USA). Between 6000 and 8000 gated events were monitored. EGFP fluorescence was monitored using flow cytometry, and the percentage of fluorescent cells was determined regardless of fluorescence intensity (Figure 1). 10T1/2 cells that were not transfected with pEGFP-N2 were used as a standard for non-fluorescence (M1). 10T1/2 cells transfected with pCI (empty vector) or pCI-skNAC without pEGFP-N2 did not display increased autofluorescence (data not shown). The number of fluorescent events (M2) was divided by the total events (M1 + M2), yielding a percentage of cells expressing EGFP. The use of EGFP as an internal control afforded the accurate calculation of transfection efficiency despite a consistent 2-fold increase in mean EGFP fluorescence in cells overexpressing skNAC, which likely reflects activation of pEGFP-N2 by skNAC (Figure 1). In addition to transfection efficiency, sample loss during processing can influence transient luciferase reporter assays. To control for this possibility, the experimental data were further normalized by the protein concentration found in each sample. Protein concentration was determined by Bradford analysis using the microassay procedure from the Bio-Rad Protein Assay system (Bio-Rad Laboratories, Hercules, CA, USA). Thus, this method of internal standardization controls for both transfection efficiency and sample loss during processing.

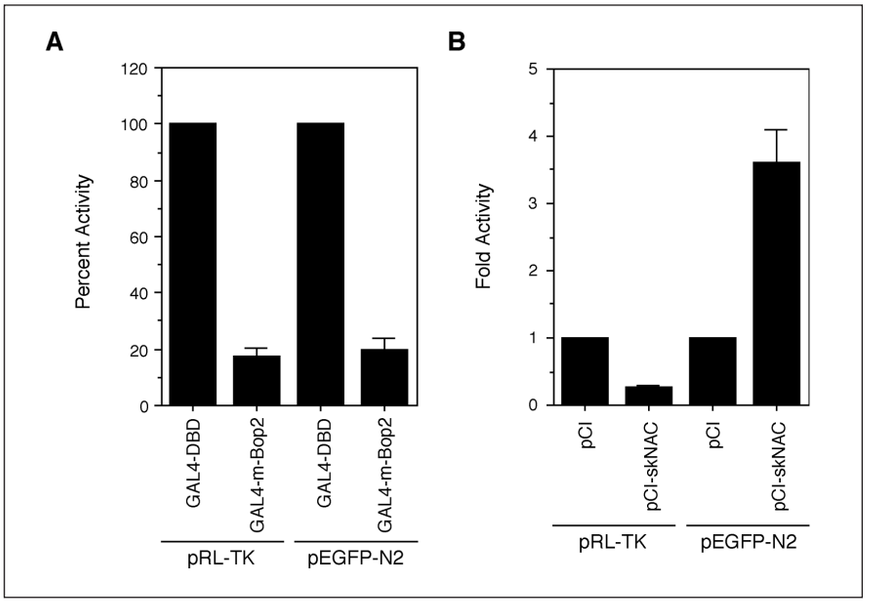

Figure 2. Comparison of pRL-TK and flow cytometry-based method for luciferase assay normalization. (A) 10T1/2 cells were transiently transfected with GAL4-DBD (2 µg) or GAL4-m-Bop2 (2 µg) along with the 5XGAL4-SV40-firefly luciferase reporter (1 µg) and the *Renilla* luciferase expression vector pRL-TK (0.5 µg) or pEGFP-N2 (0.5 µg). (B) Transient transfections of 10T1/2 cells were performed using pCI (empty vector, 3 µg) or pCI-skNAC (3 µg) together with pGL2-myoglobin (1 µg) and the *Renilla* luciferase expression vector pRL-TK (0.5 µg) or pEGFP-N2 (0.5 µg). Firefly luciferase values (experimental) were normalized by *Renilla* luciferase expression or transfection efficiency (EGFP expression monitored by flow cytometry) and protein concentration. Three independent experiments were performed to calculate the mean and standard error. pCI-skNAC and pGL2-myoglobin were a kind gift from the laboratory of R. St. Arnaud.

To determine the validity of our proposed normalization method, a comparison was made to the commonly used Dual-Luciferase Reporter Assay System (pRL-TK) under conditions that do not influence the internal control. m-Bop2 functions as a histone deacetylase-dependent transcriptional repressor (6). Additionally, m-Bop2 does not affect *Renilla* luciferase expression from the pRL-TK internal control plasmid (data not shown). The ability of a GAL4-m-Bop2 fusion protein to repress transcription was observed using an SV40-luciferase reporter construct that contains five copies of the GAL4-UAS (6). The transcriptional repression by GAL4-m-Bop2 was nearly identical when normalizing by *Renilla* luciferase expression (pRL-TK), or transfection efficiency (EGFP expression monitored by flow cytometry) and protein concentration (Figure 2A). These results suggest that the flow cytometry-based method of internal standardization described here is equally suitable for luciferase normalization when compared to the commonly used Dual-Luciferase Reporter Assay System.

Under many conditions, the commonly used standardization methods are suitable for luciferase reporter normalization. However, it is important to monitor the fluctuations of internal control gene expression when studying factors that may alter transcriptional activity within a cell. Ignoring internal control fluctuations may lead to misinterpretation of experimental values. As shown in Figure 2B, when the transcriptional regulatory potential of skNAC on the myoglobin promoter was tested, the nature of normalization had extreme effects on the perceived experimental results. When standardizing to *Renilla* luciferase expression from pRL-TK, skNAC apparently repressed luciferase expression under the control of the myoglobin promoter (pGL2-myoglobin) (Figure 2B). However, normalizing by transfection efficiency (flow cytometry-based) and protein concentration revealed that skNAC activated the myoglobin promoter, as has been previously reported (5).

It is likely that alterations of normalization controls by putative transactivators or repressors in reporter assays are not a rare event. For example, the functions of many cellular transcription factors and viral gene products are commonly studied in co-transfection assays in which the internal control plasmid contains a promoter susceptible to the factor being studied (2,4). Additionally, select biologically active compounds such as hormones or inhibitor molecules have also been shown to influence the expression of internal controls (3). In contrast to commonly used internal standards measuring cumulative enzymatic activity for normalization, the use of transfection efficiency determined by EGFP fluorescence in conjunction with protein concentration eliminates the problems that arise from internal control activation that can greatly affect the perceived experimental values. In addition to experimental conditions described in this report, the present method should prove valuable when comparing the activity of a promoter in different cell types. In experiments of these kinds, the activity of the internal control plasmid is often different in each cell type. By normalizing these experiments by transfection efficiency as determined by flow cytometry, one can directly compare the activity of a promoter in distinct cellular environments.

Because of the additional manipulations required for normalization by EGFP expression, normalization using pRL-TK is likely to be preferred under conditions where the internal control is not altered by co-transfected reagents. Under conditions that significantly repress reporter gene expression from the internal control plasmid, it is probable that normalization by measuring EGFP expression would suffer from the same problems as traditional enzymatic assays. Nevertheless, the normalization method described here provides a reliable method for normalizing transient reporter assays under a wide variety of conditions, including those in which internal enzymatic controls are unsuitable.

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