New High-Throughput Technology for Screening Transcriptional Regulatory Elements and Gene Networks

INTRODUCTION
The post-genome era has led to great advances in high-throughput genomics studies. Genomic approaches such as microarray expression analysis and ChIP-chip transcription factor binding assays have yielded new insights into genetic pathways. These technologies provide valuable observational data but do not identify the functional connections within networks or explain the mechanism of gene regulation. Transient reporter gene experiments like functional promoter assays provide an important additional layer of data for understanding mechanisms of regulation in genetic networks.

SwitchGear Genomics and Promega have combined their technologies and expertise to create a new set of tools that enable a detailed analysis of regulatory pathways in living cells. By cloning into the Promega pGL4.11[3Luc2P] Vector, SwitchGear has produced a library of thousands of human promoters, UTRs and other regulatory elements encompassing many different disease-related pathways that are available as ready-to-use tools for cell-based studies.

Some of our previous work in studying the function of 1% of the promoters in the human genome in 14 diverse cell lines was an important first step in the functional annotation of the genome (Figure 2, from Cooper et al. 2006). This comprehensive functional survey demonstrates the widespread use of alternative promoters and the strong constitutive activity of Cpg-rich promoters. We showed a strong correlation between promoter activity and the corresponding endogenous RNA transcript levels. There are interesting patterns of cell type-specific promoter

Figure 1. Integrated measures of promoter activity, transcript levels and transcription factor binding for 96 human genes. Three types of data were gathered for 96 human genes in the HCT116 human liver cell line: 1) transcript levels of the endogenous human genes measured by quantitative RT-PCR are shown on the X axis, 2) binding of the TAF1 basal transcription factor as measured by chromatin IP is shown on the Y axis, and 3) functional promoter activity measured by a promoter-luciferase reporter experiment is shown with bubbles, where the diameter of the bubble is equal to promoter activity. The genes labeled A are examples of genes with high promoter activity and high endogenous transcript levels but no detectable TAF1 binding. The genes labeled B are examples of genes with medium promoter activity and high TAF1 binding but low endogenous transcript levels.
activity and many functional alternative promoters that regulated distinct gene isoforms. This was the first experimental quantitative estimate of promoter contribution to gene regulation on a large scale.

The goal of SwitchGear Genomics is to enable groups to measure the function of thousands of regulatory elements in living cells under experimental conditions of interest. SwitchGear's functional tools have been used successfully to identify new members of gene networks and have also been used as efficient and cost-effective screening tools to detail how compounds affect entire pathways rather than just a single marker. These new screening tools will enable researchers to greatly enrich existing genomic data sets and provide functional annotation that will thoroughly describe the mechanism of action of a particular regulatory pathway.

**MATERIALS AND METHODS**

**Library creation.** Using the transcription start sites (TSSs) found in the SwitchGear database, SwitchDB (www.switchdb.com), PCR primers were designed to amplify ~1kb fragments representing promoters from the human genome. Fragments may include 800bp to 1200bp upstream of the TSS and up to 100bp downstream of the TSS (while avoiding the inclusion of the endogenous translation start site). The promoter sequences were cloned immediately 5’ to the luciferase reporter cassette of the Promega pGL4.11[luc2P] Vector using a high-throughput cloning strategy. Purified plasmids were verified by sequencing and ensured to have A260/A280 ratios greater than 1.75.

**Transfection.** HT1080 cells (ATCC) were grown and seeded in Advanced MEM (Invitrogen) with 10% FBS, 100µg/ml penicillin, 100µg/ml streptomycin. The cells were seeded at 3,750 cells/well in 100µl total volume or 1,500 cells/well in 30µl total volume (96- and 384-well formats, respectively) in white tissue culture-treated, multwell plates and incubated for 24 hours prior to transfection at 37°C in 5% CO2. For 96-well transfections we mixed 1.67µl experimental plasmid (30ng/µl), 0.3µl FuGENE® -6 transfection reagent (Roche), and 3.03µl Opti-MEM® medium (Invitrogen) and incubated for 30 minutes at room temperature before adding to the cells. For 384-well transfections, we conducted the same procedure with 1µl plasmid (30ng/µl), 0.12µl Fugene®-6, 1.88µl Opti-MEM® medium.

For the control construct experiment, we incubated the transfected cells for 24 hours at 37°C in 5% CO2 before conducting the assay. For the hypoxia experiment, we incubated the transfected cells for 8 hours and then added DFO (DFO is a known activator of HIF1α) to a final concentration of 100µM (Sigma) and continued incubation for 3, 6, 9, 24 and 34 hours before conducting the assay for luciferase activity. Each transfection and assay experiment was conducted with four replicate wells.

**Luciferase assay for activity.** To assay luciferase activity in each well, we added 100µl or 30µl (96- and 384-well formats, respectively) of Steady-Glo® Luciferase Assay Reagent (Cat.# E2520) directly to the well of transfected cells and incubated for 15 minutes at room temperature with shaking. We then used the LMax384 luminometer (Molecular Devices) to read the luminescence signal from each well for 2 seconds. For the control panel, we normalized the raw data by the average activity of the random control constructs to yield promoter activity as a fold increase over the average strength of the random control construct fragments. For the hypoxia experiment, we calculated the fold induction in activity over that of the promoter at timepoint zero.

**RESULTS**

Ensuring the reproducibility of data is very important when scaling cell-based functional promoter assays for high-throughput studies. In one study, we transfected
six replicates of a panel of 8 control constructs (four known promoters and four random genomic fragments) into human HT1080 cells. These fragments were assayed in a luciferase-based vector in 384-well format. The results of the promoter assays are highly reproducible with a low (and consistent) coefficient of variance (CV) associated with each of the eight constructs (Figure 3). This experiment also serves to demonstrate the large dynamic range of promoter activity extending almost five orders of magnitude using the luciferase reporter. Both the positive and random control fragments were chosen to represent the range of activity observed for actual human promoters and randomly sampled genomic fragments, respectively. For this experiment, we wished to measure the signal variability of replicate promoter reporter assays and therefore did not include a transfection control. When performing experiments in different cell lines or different conditions, it is often important to use a transfection control or cell viability assay. A similar experiment in 96-well format yields similar results (data not shown).

In a second study, we transfected four independent preparations of a luciferase construct containing the promoter of the lactate dehydrogenase A gene (LDHA). LDHA is known to be induced by hypoxia due to activation by HIF1α. The activity the LDHA promoter was measured during a time course of DFO exposure. These induction data are shown in Figure 4. The hypoxia induction experiment shows that functional promoter assays conducted in high-throughput format are also capable of measuring induction events over a time course and show consistent results between independent construct preparations and over a broad dynamic range. This experiment is an example of how such time course experiments may be conducted with SwitchGear tools in a high-throughput format that easily may be extended to include hundreds of promoters of interest.

CONCLUSIONS
High-throughput functional promoter assays are an integral part of any genetic network or pathway study. The data shown here highlight the ability to gather highly reproducible data over a broad dynamic range and the ability to scale this approach to hundreds or thousands of promoters in a single experiment.

REFERENCE

THE SWITCHGEAR GENOMICS LIBRARY
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