

## AN OPTIMIZED SYSTEM FOR STUDYING TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL GENE REGULATION

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Here we describe a two-part functional system for analyzing the transcriptional and post-transcriptional regulation of genes. The first part involves promoter-reporter constructs and protocols for measuring the function of human promoter elements on a large scale in living cells. The second part involves a similar concept using reporter-UTR constructs to examine the functional effect of UTR sequences on post-transcriptional regulation on a similar scale. Pairing the two types of experiments for hundreds or thousands of genes of interest will enable researchers to gain new insights into the regulatory mechanisms behind gene expression networks and pathways.

### Introduction

The relatively recent ability to study gene regulation on a genome-wide scale, such as with expression microarrays and ChIP-chip, has yielded new insights into genetic pathways and networks. These technologies provide observational data but leave gaps in network and pathway knowledge such as identification of the functional elements that affect the mRNA levels measured on a microarray. For instance, if a group of mRNAs show an increase in steady-state levels after induction with a particular compound, a researcher will be left with questions about what caused the change in steady-state levels. Is the difference a result of changes in transcriptional regulation or mRNA stability, or a combination of both? For example, the untranslated regions (UTRs) of mRNAs can play a major role in regulating translation, so does the change in steady-state transcript level affect total protein expression? To maintain high-throughput momentum in gene expression studies with the goal of understanding mechanisms of change, researchers need the ability to understand the function of transcriptional and post-transcriptional regulatory elements on a similarly large scale.

**A number of recent studies have highlighted the importance of the combination of 3' UTRs and miRNAs in gene regulation.**

Promoter-reporter assays have been a standard approach for studying transcriptional regulatory element function for many years. However, only in the last few years has this approach been applied to studying hundreds of promoters in a single experiment (1). Though studies of the function of UTR sequences using hybrid reporter-UTR assays have been used for a handful of genes, a gap exists in the tools available for studying these elements on a large scale. While the function of 5' UTRs in gene regulation is undoubtedly important, the 3' UTR's apparent length flexibility, and possible (highly speculative) correlation with increasing

organism complexity suggests that studies of the 3' UTR will also contribute to understanding gene regulation (2,3). A number of recent studies have highlighted the importance of the combination of 3' UTRs and miRNAs in gene regulation. For example, more than 40% of miRNAs are estimated to "match" conserved motifs in 3' UTRs (4). At SwitchGear our ultimate goal is to study both classes of UTR, and we have started by examining the function of 3' UTRs.

### Transcriptional and Post-Transcriptional Regulatory Control

The literature describes a handful of genes for which studies of the function of both transcriptional and post-transcriptional regulatory elements have been carried out, and in all cases such studies have yielded valuable insight into that gene's regulation. One of the best characterized is the human transferrin receptor gene. Human transferrin receptor (TFRC) levels increase more than 10-fold when the iron-chelator deferoxamine (DFO) is added to cells in culture to induce iron depletion. These increased levels of TFRC are influenced by both the promoter and 3' UTR region (5,6). A second example of a gene studied at the transcriptional and post-transcriptional stages is  $\beta$ -globin. Early mutagenesis studies of the  $\beta$ -globin promoter indicated the importance of promoter function in the proper regulation of the locus (7), and studies in subsequent years have revealed naturally occurring mutations in globin promoters that are associated with thalassemias (8,9). In addition, globin family mRNAs are known to be exceptionally stable, and functional studies of globin 3' UTRs have revealed motifs essential to this stability (10). A final example is the cyclin D1 (CCND1) locus, which often is involved in hematopoietic malignancies and is known to cause a neoplastic pathology when overexpressed. Overexpression of CCND1 can result from either one, or the additive effect of two, independent genetic events. In the first event, a common translocation places the transcription start site of the CCND1 locus within range of a strong transcriptional enhancer. In the second event, rearrangements and deletions in the CCND1 3' UTR, often associated with genomic instability, act to increase the half-life of the mRNA (11).

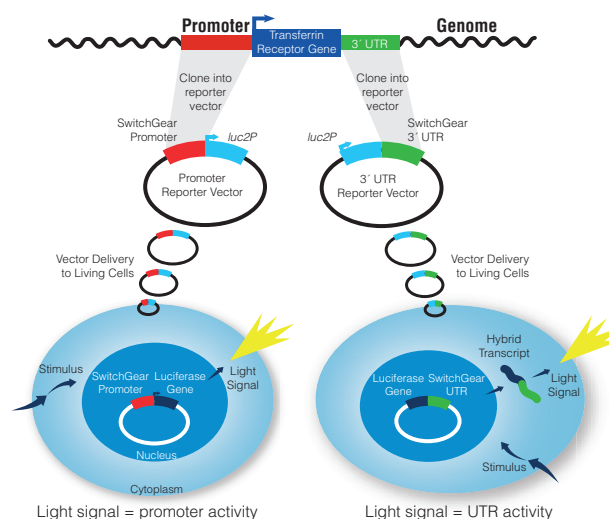
## A System for Studying Gene Regulation on a Large Scale

Undoubtedly, these few examples represent only a fraction of genes for which in-depth studies of transcriptional and post-transcriptional functional elements will lead to a better understanding of regulatory mechanisms. Loci with interesting behavior on expression microarrays represent some of the best candidates for follow-up studies. In order to facilitate these follow-up studies, we created a two-part functional study system. The first part involves promoter-reporter constructs and protocols for measuring the function of human promoter elements on a large scale in living cells. The second part involves a similar concept using reporter-UTR constructs to examine the functional effect of UTR sequences on post-transcriptional regulation on a similar scale. By pairing the two types of experiments (transcriptional and post-transcriptional) for hundreds or thousands of genes of interest, we will enable researchers to gain new insights into the regulatory mechanisms behind gene expression networks and pathways.

We have spent the past few years perfecting promoter prediction across the human genome along with high-throughput cloning and cell-based assay techniques to enable functional promoter-reporter assays on a large scale. Using this strategy we have made a survey of functional promoter activity over 1% of the human genome, and we have conducted large-scale experiments measuring activity of promoters representing entire pathways before and after activation of the pathway (1,12); therefore, the first piece of the large-scale functional assay is in place. To move forward, our goal is to create a vector assay system and library of regulatory elements for the high-throughput functional analysis of 3' UTRs.

## Insight into the Transcriptional and Post-Transcriptional Regulation of the Human Transferrin Receptor (TFRC)

We created an UTR functional assay vector using a modified firefly luciferase gene (*luc2P*; technology licensed from Promega) driven by a constitutive promoter (RPL10) with a multiple cloning region between the stop codon and the polyA signal for *luc2P*. The constitutive promoter drives production of a hybrid *luc2P*-human 3' UTR transcript. Therefore, the total luciferase output depends on the effect of the 3' UTR on the hybrid transcript's stability and/or translation efficiency. This vector design is compatible with our current high-throughput cloning strategies, and we have started constructing a library of thousands of human 3' UTR sequences. As an example of studying both promoters and UTRs with a standard reporter output, we chose to examine TFRC. As discussed previously, TFRC protein levels are known to increase significantly (more than tenfold) in cells that are subjected to iron depletion. Previous researchers have found that the TFRC promoter activity increased ~2.8-fold upon treatment with DFO (5). The role of post-



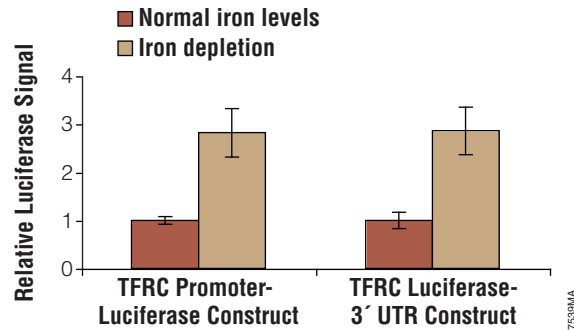
**Figure 1. The design of constructs used to gain an insight into the transcriptional and post-transcriptional regulation of the human transferrin receptor (TFRC).** In the first construct, a 944 bp TFRC promoter sequence (red) was cloned upstream of a modified Promega firefly luciferase gene (*luc2P*). In the second construct, a 2437 bp TFRC 3' UTR (green) was cloned into a multiple cloning region downstream of the *luc2P* cassette. The RPL10 promoter drives constitutive expression of the hybrid construct. HT180 cells (human fibrosarcoma) were transfected with each construct.

transcriptional regulation was elucidated by the construction of a TFRC “minigene” on a plasmid, showing that when the entire 3' UTR is deleted, the expression response to DFO is decreased by ~3-fold (6).

Our goal was to test the same transcriptional and post-transcriptional regulatory elements in our two-part system. We cloned a ~1 kb sequence representing the TFRC promoter upstream of our luciferase reporter cassette. On a second construct, we cloned a ~2.5 kb fragment representing the entire TFRC UTR downstream of our luciferase reporter cassette such that a hybrid luciferase-TFRC UTR mRNA was produced (Figure 1). We transfected each construct into HT180 cells (human fibrosarcoma) in a 96-well format and assayed each construct's activity before and after the addition of 100  $\mu$ M DFO. As shown in Figure 2, the results we observed are significant (t-test  $p < .05$ ) and are in line with what we expected based on previous published reports. TFRC represents a case in which studying the function of both the transcriptional and post-transcriptional regulatory elements is indispensable for gaining a complete understanding of the gene's regulation.

## Methods

The 3' UTR reporter vector was constructed by cloning the human RPL10 promoter (ChrX: 153146645-153147658) into a multiple cloning region upstream of the firefly luciferase (*luc2P*) reporter cassette followed by inserting a new multiple cloning region between the *luc2P* stop codon and polyA



**Figure 2. Iron depletion results in increased rates of transcription (regulated by the promoter) and increased transcript stability (regulated by the 3' UTR), both contributing equally to the overall accumulation of the TFRC transcript.**

signal. For the TFRC experiment, we constructed the promoter reporter vector by inserting a 944 bp TFRC promoter sequence upstream of the *luc2P* reporter (Chr3: 197297200-197298143). To assay the TFRC 3' UTR, we cloned a 2,437 bp UTR sequence into the multiple cloning region downstream of the *luc2P* cassette (Chr3: 197264668-197267104). All sequence coordinates are for the May 2004 freeze (genome.ucsc.edu).

For the cell-based assays, we first seeded approximately 5,000 HT1080 human fibrosarcoma cells (ATCC #CCL-121) per well in a 96-well format in standard growth conditions. Twenty four hours after seeding, we transfected 50 ng of

plasmid DNA per well with FuGene®-6 (Roche Cat.# 11814443001) according to the manufacturer's protocol. Approximately 16 hours post-transfection, we added DFO to the experimental wells to a final concentration of 100 μM (Sigma Cat.# D9533). We transfected each construct in triplicate in each condition (treated and untreated). Finally, twenty-four hours after induction, we assayed each well using 100 μl of Steady-Glo® Luciferase Assay Reagent (Cat.# E2520) reading for two seconds on a standard plate luminometer (Molecular Devices LMax384).

This experiment was designed to assay the effect of a small molecule (DFO) on transcriptional and post-transcriptional regulatory elements. We had no prior information on the effect of DFO on the promoters that drive *Renilla* expression from plasmids normally used as transfection controls. Therefore, we conducted the experiment with a single reporter to avoid confounding factors, and we performed replicate transfections to assess well-to-well variability in transfection efficiency.

**Summary**

High-throughput studies of both transcriptional and post-transcriptional regulatory elements will become increasingly important as we continue to gather genome-scale expression and transcription factor binding data. This two-part reporter system and our libraries of thousands of human promoters and 3' UTRs will enable these studies on an unprecedented scale.

**References**

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**Promega Protocols**

*pGL4 Luciferase Reporter Vectors Technical Manual #TM259* ([www.promega.com/tbs/tm259/tm259.html](http://www.promega.com/tbs/tm259/tm259.html))

*Steady-Glo® Luciferase Assay System Technical Manual #TM051* ([www.promega.com/tbs/tm051/tm051.html](http://www.promega.com/tbs/tm051/tm051.html))

**SwitchGear Genomics Protocols**

SwitchGear Genomics Transfection Protocol (<http://switchgeargenomics.com/technical/protocols/adherentcells.html>)

**Ordering Information**

Product	Size	Cat.#
Steady-Glo® Luciferase Assay System	10 ml	E2510

**Product**

SwitchGear Genome-Wide Promoter Library in Luciferase Vector (<http://switchgeargenomics.com/product/promoterlibrary.html>)

SwitchGear Genome-Wide UTR Library in Luciferase Vector (<http://switchgeargenomics.com/product/utrlibrary.html>)

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