A Novel System for Regulated Protein Expression in Mammalian Cells

ABSTRACT We describe the Regulated Mammalian Expression System, which allows researchers to achieve tightly controlled protein expression in mammalian cells. This system uses coumerin-based compounds to modulate regulation of gene expression while maintaining high levels of induction over low levels of basal activity. In addition, expression of the gene of interest can be turned off rapidly by novobiocin addition. Introduction of a unique auto-amplification loop for transactivator expression makes the system robust and minimizes potential undesired effects on host gene expression.

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Expression levels are directly correlated to concentrations of coumermycin and can be rapidly switched off by adding novobiocin.

INTRODUCTION

Regulated expression of recombinant proteins in mammalian cells is an important attribute for analysis of protein function and production. Expression of a gene of interest in a dose-responsive manner allows conditional modulation of the timing and level of transgene expression in cells. This provides more physiologically relevant experimental systems.

Many examples of the successful application of inducible mammalian expression systems have been reported (1–4). Those systems have been aimed mainly at maximizing the ratio between the levels of transgene expression in the induced, or "on", state and the level of background transgene expression with no induction, or "off" state. However, these systems have not achieved some features that are desirable in a regulated expression system, such as a rapid on-off switch and the minimizing of a "squelching" effect due to overexpression of strong activators.

HOW THE REGULATED MAMMALIAN EXPRESSION SYSTEM FUNCTIONS

The Regulated Mammalian Expression System[a–e] (Cat.# C9470) relies upon two specially designed plasmids that are co-transfected or stably integrated into mammalian cells, and the modulator compounds, coumermycin and novobiocin, which are added directly to cell culture medium (Figure 1; 5). The system uses the pF12 RM Flexi® Vector, a specialized vector designed for regulated mammalian protein expression. This vector is compatible with the Flexi® Vector System, which allows rapid, efficient and high-fidelity transfer of protein coding sequences within a suite of vectors containing different expression options.

The Regulated Mammalian Expression System works by cloning the protein coding sequences of interest into a pF12 RM Flexi® Vector. This vector is co-transfected into mammalian cells with the

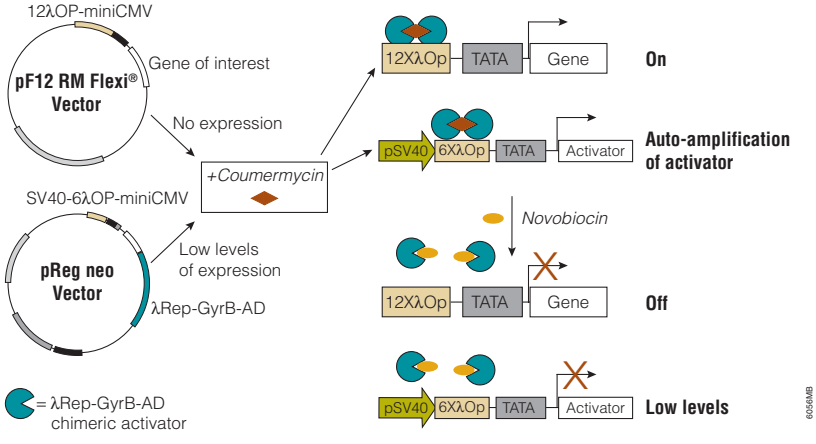

Figure 1. Schematic representation of the coumerin-regulated system. Based on the gene expression system that was developed in the laboratory of Dr. Shi-Hsiang Shen (5).

pReg neo Vector[b–d]. Once inside cells, the pReg neo Vector maintains expression of a chimeric transactivator at low basal levels from a hybrid promoter comprising SV40 early, λ operator consensus (6λOP) and minimal CMV (mini-CMV) sequences. The chimeric transactivator is composed of three parts: i) a λ repressor DNA binding domain (λRep), which binds λ operator sequences; ii) a bacterial gyrase B subunit domain (GyrB); and iii) an NF-κB p65 transcriptional activation domain (AD; Figure 1). Addition of coumermycin drives transactivator homodimerization via the GyrB domain and promotes binding by the dimerized λ repressor domains to the λ operator sequences in the promoter region of the pF12 RM Flexi® Vector (12λOP-miniCMV).

Once the p65 NF-κB activation domain is positioned in the promoter region, transcription of the downstream gene of interest is increased. Furthermore, the hybrid promoter on the pReg neo Vector is designed to function in a positive feedback loop. Homodimeric transactivator triggers additional transcription of the chimeric transactivator by binding to the λ operator consensus sequences within this hybrid promoter, providing more transactivator molecules for increased regulated gene expression. The expression can be rapidly switched off by adding novobiocin. Novobiocin resembles a monomeric version of coumermycin in structure and effectively competes and abolishes transactivator dimerization and coumermycin-induced protein expression (6,7).

WIDE DYNAMIC RANGE OF INDUCTION

To characterize performance of the Regulated Mammalian Expression System we used HEK293 stable cell lines expressing the reporter gene, firefly luciferase. We chose firefly luciferase as our protein of interest because of the exquisite sensitivity and wide dynamic range of detection that bioluminescent assays afford. Stable clones expressing both chimeric transactivator and firefly luciferase under coumermycin-dependent inducible promoters were generated and selected using the antibiotic G-418. Antibiotic resistant clones were then screened using luciferase assays for desired characteristics of low basal activity and high induction ratio in the presence of coumermycin. Figure 2 depicts firefly luciferase expression upon addition of increasing amounts of coumermycin. In the absence of coumermycin, negligible luciferase activity was measured, corresponding to less than 10 molecules per cell.

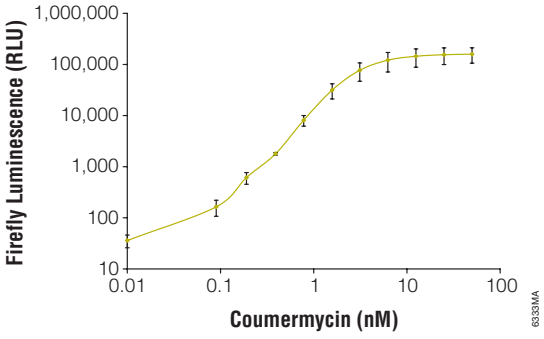

Figure 2. Protein induction in HEK293 stable cell line. An HEK293 stable cell line was generated using vectors containing firefly luciferase under the regulated vector and the regulated chimeric transactivator. Cells from this cell line were plated in a 96-well plate at 10,000 cells/well. To induce firefly luciferase expression, increasing concentrations of coumermycin were added directly to the medium. Firefly luciferase activity was measured 24 hours after induction using the Bright-Glo™ Luciferase Assay System (Cat.# E2650). Each data point represents replicate samples from four wells. Relative light units (RLU) measured in non-induced cell samples were similar to the luminometer instrument background levels. The instrument background mean RLU value was subtracted from each sample.

The induction of luciferase expression by coumermycin was dose-dependent between 0.1 and 5nM concentrations. Apparent induction of luciferase expression was detected following addition of coumermycin at concentrations as low as 0.1nM. The level of expression further increased (~1,000 fold) with increasing coumermycin concentrations, reaching a maximum at ~5nM coumermycin. The changes in luciferase activity upon induction were rapid, and detectable within a few hours, reaching a maximum after 24 hours of induction (data not shown). These data demonstrate that this system allows generation of stable cell line clones with highly regulated protein expression. Luciferase expression was not detected in the non-induced state and showed linear protein expression over a fairly broad range of coumermycin concentrations.

Induction
of luciferase expression by coumermycin was dose-dependent and reached a maximum of ~1,000-fold increase at ~5nM of coumermycin.

We also tested performance of the Regulated Mammalian Expression System in multiple cell lines, including HeLa, CHO, U2OS, K-562, and COS-7. Stable cell lines were generated by simultaneous co-transfection of both transactivator and gene expression vectors without first establishing a transactivator "master cell line". In these cell lines, stable integration of a bioluminescent protein under coumermycin-inducible regulation allowed us to select clones with extremely low or undetectable levels of basal activity and more than a 1,000-fold induction following coumermycin addition.

Figure 3 shows the dose-dependent expression of TMR-labeled DRD1-HaloTag® receptor upon coumermycin addition. For this study, the dopamine receptor was cloned into the pF12A RM Flexi® Vector as a C-terminal fusion partner with the HaloTag® protein. HaloTag® protein covalently binds specially designed chloroalkane ligands such as the HaloTag® TMR Ligand to allow fluorescent monitoring of the HaloTag® protein partner in cells and in vitro (8). Similar to the firefly luciferase results (Figure 2), levels of regulated expression were dependent upon coumermycin concentration and showed a dose-dependent increase with 0.1 to 5nM concentrations of coumermycin.

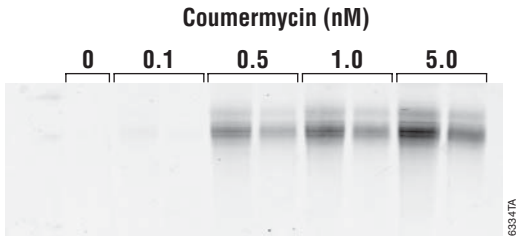

Figure 3. Dose-dependent expression of dopamine (DRD1) receptor fused with HaloTag® protein in a CHO stable cell line. CHO cells were co-transfected with pReg neo Vector and pF12A RM Flexi® Vector containing a DRD1-HaloTag® fusion clone. Both vectors were linearized with XmnI and BstBI restriction enzymes, respectively, prior to transfection. Stable cell lines were generated using G-418 selection. The expression levels of DRD1-HaloTag® fusion receptor in the stable cell lines were visualized by staining cells with fluorescently labeled HaloTag® TMR Ligand (Cat.# G8251). Cells expressing high levels of the fusion protein 24 hours after induction with 5nM Coumermycin were separated by fluorescence activated cell sorting (FACS®) analysis and propagated. The positive FACS® sorted cell population was exposed to increasing amounts of coumermycin, and 24 hours after induction with coumermycin, the cells were stained with HaloTag® TMR Ligand, and the amount of labeled fusion protein was determined by SDS-PAGE analysis. For each coumermycin concentration, cell lysate corresponding to 2×10^4 and 0.5×10^4 cell equivalents were loaded on the gel. Lane 0, non-induced cells.

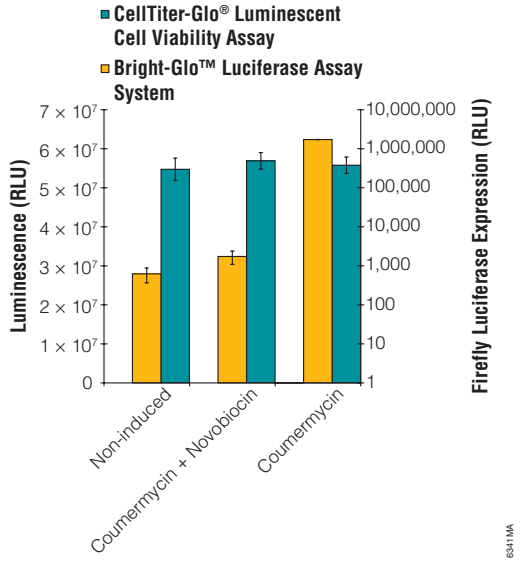

Figure 4. Coumermycin-induced protein expression is inhibited by novobiocin. Cells from the HEK293 stable cell line described in Figure 2 were plated in 12-well plates at 100,000 cells/well. The cells either received no treatment (noninduced), were treated with coumermycin at a final concentration of 5nM, or were treated with 5nM coumermycin plus 10µM novobiocin. The cells were collected and analyzed for firefly luciferase expression and cell viability 24 hours after treatment. Each data point represents four replicates. Firefly luciferase activity was measured in cell lysates using Bright-Glo™ Luciferase Assay System (Cat.# E2650). Cell viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570).

MODULATOR COMPOUNDS: NOVOBIOCIN AS AN "OFF" SWITCH

A unique characteristic of the Regulated Mammalian Expression System is the ability to rapidly shut down coumermycin-induced protein expression by novobiocin, a competitive inhibitor of coumermycin. To illustrate this, we compared coumermycin-induced expression levels of firefly luciferase in the presence or absence of novobiocin. In the presence of excess novobiocin, we effectively inhibited (more than 99%) coumermycin-induced firefly luciferase expression. No decrease in cell viability was observed 24 hours after compound addition (Figure 4).

The kinetics for the novobiocin "switch off" of protein expression were examined in a stable line of CHO cells expressing regulated *Renilla* luciferase activity. The changes in *Renilla* luciferase activity after novobiocin addition were followed over time. Figure 5 shows *Renilla* luciferase activity increased in samples not treated with novobiocin, while the addition of novobiocin resulted in a decrease of *Renilla* luciferase activity that correlated well with the *Renilla* luciferase half-life (~5.5 hours). The decrease in *Renilla* luciferase activity was rapid, was detected as soon as 4 hours after novobiocin addition and was reduced to less than 80% after 12 hours.

Adding excess novobiocin effectively inhibited more than 99% of coumermycin-induced firefly luciferase expression.

Both coumermycin and novobiocin compounds have excellent pharmacokinetic properties that make them well adapted for in vivo use. Both compounds bind bacterial GyrB with high affinity (K_d = 3–5 × 10^{-8}M) (9), and their half-lives in animals and serum are reported as 5.5 to 6 hours (10,11). In addition, these compounds are specific for the bacterial form of GyrB, and no known targets exist for high-affinity binding in mammalian cells.

TRANSIENT EXPRESSION PERFORMANCE

In transient transfections, performance of the Regulated Mammalian Expression System depends on the basal activity of the minimal CMV promoter upstream of the transgene for the protein of interest, the activity of the SV40 hybrid promoter driving expression of the transactivator protein. At high levels the transactivator protein may homodimerize even in the absence of coumermycin. This leads to increased expression of the coding sequences for the gene of interest. Basal activities of the promoters for the transactivator and transgene will vary with cell type and transfection efficiency. Therefore, it is important in transient expression experiments to consider the synergistic nature between transactivator expression and availability of the regulated promoter for activation.

We characterized regulated protein expression under transient conditions in several cell lines using bioluminescent reporters. Basal activity of the minimal CMV promoter was low in HeLa and COS-7 cells, 1.5- to 2-fold above assay background (Figure 6). By comparison, basal activity from the minimal CMV promoter was more than 10-fold higher in HEK293 cells using identical transfection conditions. Basal activity in HEK293 cells was reduced to near background levels by lowering the total amount of plasmid DNA transfected by 10-fold.

SUMMARY

The new Regulated Mammalian Expression System incorporates features that are essential for controlled expression of proteins in mammalian cells. These include tight, specific and rapid "On" and "Off" regulation that is

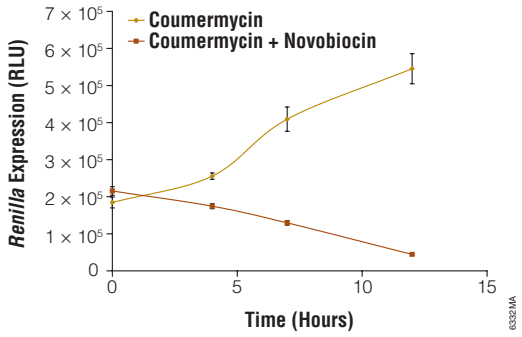

Figure 5. Novobiocin rapidly terminates coumermycin-induced protein expression. Cells from the CHO stable cell line expressing regulated *Renilla* luciferase were plated in a 96-well plate. EnduRen™ Live Cell Substrate (Cat.# E6481) and 5nM coumermycin (final concentration) were added to the cell culture medium. Fourteen hours after plating, the cells were assayed for *Renilla* luciferase activity (0 time point). Then 10µM novobiocin was added to selected wells. The entire plate was read at indicated time points to determine changes in *Renilla* luciferase expression levels. Each data point represents twelve replicates.

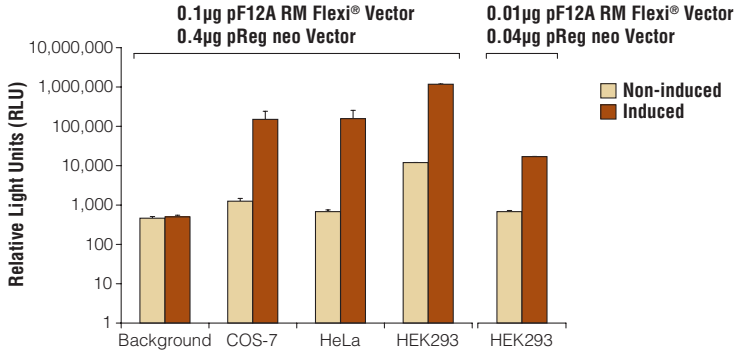

Figure 6. Effect of the total amount of pF12A Flexi® and pReg neo Vectors used for transfection. COS-7, HeLa and HEK293 cells were plated in 12-well culture dishes at 10^6 cells/well and transfected with a total of 1µg of DNA. Each DNA sample for transfection included a 1:4 ratio of the pF12A RM Flexi® Vector with a firefly luciferase coding sequence (0.1µg or 0.01µg in the 10% sample) to pReg neo Vector (0.4µg or 0.04µg in the 10% sample) plus 0.2µg of phRL-SV40 DNA. Carrier DNA was added so that the final amount of DNA was 1µg. Three hours post-transfection, coumermycin was added (5nM final concentration) to induce firefly luciferase expression (induced), and no coumermycin (non-induced) was added to the corresponding control wells to monitor basal activity. Twenty-four hours after coumermycin addition, cells were harvested, lysed and assayed for firefly and *Renilla* luciferase activities with the Dual-Glo™ Luciferase Assay System (Cat.# E2980). Background was measured using nontransfected cells. The firefly luciferase expression was normalized to *Renilla* luciferase as the internal transfection normalization factor and expressed as Relative Light Units.

dose-dependent, high ratios for protein induction, and modulator compounds that are not toxic. Vector elements are designed to produce low basal levels of protein expression and high expression levels following induction with coumermycin. Protein expression levels are directly correlated with coumermycin concentrations ranging from 0.1 and 5nM. Novobiocin rapidly shuts down expression of the induced protein, providing another level of control for analysis of protein function with mammalian cells. Both compounds are relatively nontoxic at the concentrations used with the system and are well-suited for in vivo applications.

The pReg neo Vector included with the system encodes the chimeric transactivator and neomycin phosphotransferase gene for stable cell selection. The pF12 RM Flexi® Vector component is available as both ampicillin- and kanamycin-resistant plasmids. The pF12 RM Flexi® Vectors offer the flexibility to transfer the protein coding region of interest to other Flexi® Vectors, which offer different protein expression and application options. Although the system can be used for transient expression studies, the greatest degree of protein expression control and inducibility are obtained when generating stable cell clones.

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ORDERING INFORMATION

Product	Size	Cat.#
Regulated Mammalian Expression System I	system	C9470
pF12A RM Flexi® Vector	20µg	C9431
pF12K RM Flexi® Vector	20µg	C9441
pReg neo Vector	20µg	C9421
Coumermycin A1	5mg	C9451
Novobiocin Sodium Salt	1g	C9461

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Different ratios of the Flexi® Vector to pReg neo Vector, as well as different total amounts of vector DNAs, may be needed to optimize transient expression studies.