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.

Jolanta Vidugiriene, Ph.D., Jim Hartnett, M.S., Elaine Schenborn, Ph.D., and Michael R. Slater, Ph.D., Promega Corporation

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 (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. 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 (60.OPl) 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 gyrB 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 (12LOP-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.

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.

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 Lidant 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.

**Induction of luciferase expression by coumermycin was dose-dependent and reached a maximum of ~1,000-fold increase at ~5nM of coumermycin.**
by staining cells with fluorescently labeled HaloTag® TMR Ligand (Cat.# G8251). Cells expressing high levels of the fusion protein 24 hours after induction with SnfM Coumermycin were separated by fluorescence activated cell sorting (FACS®) analysis and propagated. The positive FACS® sorted cell population was used for transient transfection performance. To illustrate this, we compared coumermycin-induced expression levels of the fusion protein 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, as detected as soon as 4 hours after novobiocin addition and was reduced to less than 80% after 12 hours.

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 (Kd = 3–5 x 10^-10 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.

**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
Luciferase expression levels. Each data point represents twelve replicates. 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.

Novobiocin rapidly shuts down expression related with coumermycin concentrations ranging from 0.1 and 5nM. Novobiocin rapidly terminates coumermycin-induced protein 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.

Cells from the CHO stable cell line expressing the chimeric transactivator and neomycin phosphotransferase gene for stable cell selection. The pF12 RM Flex® 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 Flex® 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.

The pReg neo Vector included with the system encodes the chimeric transactivator and neomycin phosphotransferase gene for stable cell selection. The pF12 RM Flex® Vector component is available as both ampicillin- and kanamycin-resistant plasmids. The pF12 RM Flex® Vectors offer the flexibility to transfer the protein coding region of interest to other Flex® 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.

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 at indicated time points to determine changes in Renilla luciferase expression levels. Each data point represents twelve replicates.

**REFERENCES**

22. ordering information

**PRODUCT**

- **pF12A RM Flex® Vector**
- **pReg neo Vector**
- **Coumermycin A1**

**ORDERING INFORMATION**

- **pF12A RM Flex® Vector**
- **pReg neo Vector**
- **Coumermycin A1**

**REFERENCES**